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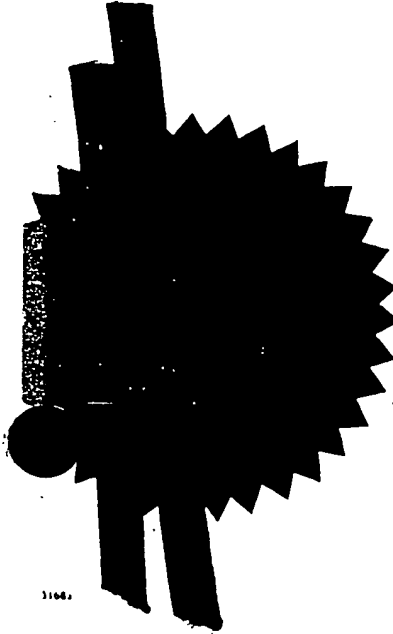
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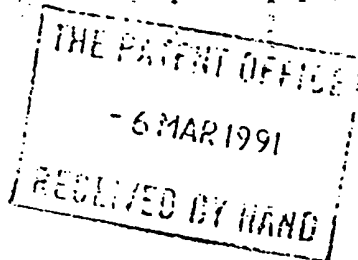
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1   Please give the title of the invention   BINDING SUBSTANCES

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First or only applicant

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   Country   U.K.

   ADP number *(if known)*   58 376790c/

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BINDING SUBSTANCES

The present invention relates to binding substances. The present invention also relates to methods for the production of binding substances eg binding molecules and to the biological binding molecules produced by these methods. The present invention also relates to: a) the production of antibodies, receptor molecules and fragments and derivatives of these antibodies and receptor molecules; b) viruses encoding the above identified molecules, which viruses have the ability to present said molecules at their surfaces; c) packages comprising a virus and an above identified molecule presented at the viral surface; and d) screening techniques utilising the unique properties of these packages.

Owing to their high specificity for a given antigen, the advent of monoclonal antibodies (Kohler, G. and Milstein C; 1975 Nature $\underline{256}$: 495) represented a significant technical break-through with important consequences both scientifically and commercially.

Monoclonal antibodies are made by establishing an immortal mammalian cell line which is derived from a single immunoglobulin producing cell secreting one form of a biologically functional antibody molecule with a particular specificity. Because the antibody-secreting mammalian cell line is immortal, the characteristics of the antibody are reproducible from batch to batch. The key properties of monoclonal antibodies are their specificity for a particular antigen and the reproducibility with which they can be manufactured.

Structurally, the simplest antibody (IgG) comprises four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulphide bonds (see figure 1). Each chain has a constant region (C) and a variable region (V). The antibody has two arms (the Fab region) each of which has a V_L and a V_H region associated with each other. It is this pair of V regions (V_L and V_H) that differ

from one antibody to another, and which together are responsible for recognising the antigen. In even more detail, each V region is made up from three complementarity determining regions (CDR) separated by four framework regions (FR). The CDR's are the most variable part of the variable regions, and they perform the critical antigen binding function. The CDR regions are derived from many potential germ line sequences via a complex process involving recombination, mutation and selection.

It has been shown that the function of binding antigens can be performed by fragments of a whole antibody. Binding fragments are the F_V fragment which comprises the V_L and V🔲 of a single arm of the antibody, and the dAb fragment (Ward, E.S. et al., Nature $\underline{341}$, 544-546 (1989); which consists of a single heavy chain variable domain (V_H).

Although the F_V fragment is coded for by separate genes, it has proved possible to construct a linker that enables them to be made as a single protein chain (known a single chain F_V (scFv); Bird, R.E. et al., Science $\underline{423}$, 423-426 (1988) Huston, J.S. et al., Proc. Natl. Acad. Sci., USA $\underline{85}$, 5879-5883 (1988)) by recombinant methods.

Whilst monoclonal antibodies, their fragments and derivatives have been enormously advantageous, there are nevertheless a number of limitations associated with them.

Firstly, the therapeutic applications of monoclonal antibodies produced by human immortal cell lines holds great promise for the treatment of a wide range of diseases (Clinical Applications of Monoclonal Antibodies. Edited by E. S. Lennox. British Medical Bulletin 1984. Publishers Churchill Livingstone). Unfortunately, immortal antibody-producing human cell lines are very difficult to establish and they give low yields of antibody (approximately 1 µg/ml). In contrast, equivalent rodent cell lines yield high amounts of antibody (approximately 100 µg/ml). However, the repeated administration of these foreign rodent proteins to humans can lead to harmful hypersensitivity reactions. In the main therefore, these

rodent-derived monoclonal antibodies have limited therapeutic use.

Secondly, a key aspect in the isolation of monoclonal antibodies is how many different antibody producing cells
5 with different specificities, can be sampled, compared to how many need to be sampled in order to isolate a cell producing antibody with the desired specificity characteristics (Milstein, C., Royal Soc. Croonian Lecture, Proc. R. Soc. London B. <u>239</u>; 1-16, (1990)). For example,
10 the number of different specificities expressed at any one time by lymphocytes of the murine immune system is thought to be approximately 10^7 and this is only a small proportion of the potential repertoire of specificities. However, during the isolation of a typical antibody producing cell
15 with a desired specificity, the investigator is only able to sample 10^3 to 10^4 individual specificities. The problem is worse in the human, where one has approximately 10^{12} lymphocyte specificities, with the limitation on sampling of 10^3 or 10^4 remaining.
20 This problem has been alleviated to some extent in laboratory animals by the use of immunisation regimes. Thus, where one wants to produce monoclonal antibodies having a specificity against a particular epitope, an animal is immunised with an immunogen expressing that epitope. The
25 animal will then mount an immune response against the immunogen and there will be a proliferation of lymphocytes which have specificity against the epitope. Owing to this proliferation of lymphocytes with the desired specificity, it becomes easier to detect them in the sampling procedure.
30 However, this approach is not successful in all cases, as a suitable immunogen may not be available. Furthermore, where one wants to produce human monoclonal antibodies (eg for therapeutic administration as previously discussed) such an approach is not practically or ethically feasible.
35 In the last few years, these problems have in part,

been addressed by the application of recombinant DNA methods to the isolation and production of antigen binding fragments of an antibody molecule in bacteria such as <u>E.coli</u>. Furthermore, the use of polymerase chain reaction (PCR)

5 amplification (Saiki, R.K., et al., Science <u>239</u>, 4387-491 (1988)) to isolate antibody producing sequences from cells and organs, has great potential for speeding up the timescale under which specificities can be isolated. Amplified V_H and V_L genes are cloned directly into vectors

10 for expression in bacteria or mammalian cells (Orlandi, R., et al., 1989, Proc. Natl. Acad. Sci., USA <u>86</u>, 3833-3837; Ward, E.S., et al., 1989 supra; Larrick, J.W., et al., 1989, Biochem. Biophys. Res. Commun. 160, 1250-1255; Sastry, L. et al., 1989, Proc. Natl. Acad. Sci., USA., <u>86</u>, 5728-5732).

15 Conversely, some of these techniques can exacerbate the screening problems. For example, large separate heavy and light chain libraries have been produced from immunized mice and combined together in a random combinatorial manner prior to screening (Huse, W.D. et al., 1989, Science <u>246</u>, 1275-

20 1281). Crucially however, the information held within each cell, namely the specific combination of one light chain with one heavy chain, is lost. This loses most, if not all, of the advantage gained by using immunization protocols in the animal. Currently, only libraries derived from single

25 heavy chain variable domains (dAbs; Ward, E.S., et al., 1989, supra.) do not suffer this drawback, but because not all antibody heavy chain variable regions are capable of binding antigen, more have to be screened.

In addition, the problem of directly screening many

30 different specificities in prokaryotes remains to be solved.

Thus, there is a need for a screening system which ameloriates or overcome one or more of the above or other problems. The ideal system would allow the sampling of very large numbers of specificities (eg of the order of 10^6 and

35 higher) rapid sorting at each cloning round, and rapid

transfer of the genetic material coding for the binding molecule from one stage of the production process, to the next stage.

The most attractive candidates for this type of screening, would be prokaryotic organisms (because they grow quickly, are relatively simple to manipulate and because large numbers of clones can be created) which express and retain antibody on their surface. It has already been shown that antibody fragments can be secreted through bacterial membranes with the appropriate signal peptide (Skerra, A., and Pluckthun, A., 1988, Science 240, 1038-1040; Better, M. et al., 1988, Science 240, 1041-1043). However, it has not been shown how an antibody or antibody fragment can be held on the bacterial cell surface in a configuration which allows efficient sampling of its antigen binding properties. In large part, this is because the bacterial surface is a complex structure, and in the gram-negative organisms there is an outer wall which further complicates the position.

Bacteriophage make attractive candidates because in general their surface is a much simpler structure, they can be grown easily in large numbers, are amenable to the practical handling involved in many potential mass screening programmes and they carry genetic information for their own synthesis within a small, simple package. The difficulty has been to practically solve the problem of how to use bacteriophages in this manner. For example, a Genex Corporation patent application number PCT/US88/00716 has proposed that the bacteriophage lambda would be a suitable vehicle for the expression of antibody molecules, but no proposals provide a teaching which enables the general idea to be carried out. For example PCT/US88/00716 does not demonstrate that any sequences: a) have been expressed as a fusion with gene V; b) have been expressed on the surface of lambda; and c) retain biological activity. Furthermore there is no teaching on how to screen for suitable fusions.

The problem of how to use bacteriophages is in fact a

difficult one. The antibody molecule must be inserted into the phage in such a way that the integrity of the phage coat is not undermined, and the antibody itself should be biologically active. Thus the antibody should fold

5 efficiently and correctly and be presented for antigen binding. However, solving the problem for antibody molecules and fragments would also provide a general method for the screening of many receptor molecules.

Surprisingly, the applicants have been able to

10 construct a bacteriophage that expresses and presents on its surface large binding molecules (eg large biologically functional antibody molecules) and which remains intact and infectious. The applicants have called the structure which comprises a virus particle and a binding molecule presented

15 at the viral surface a 'package'. Where the binding molecule is an antibody (or a fragment or derivative of an antibody), the applicants call the package a phage antibody. However, except where the context demands otherwise, when the term phage antibody is used generally it should also be

20 interpreted as referring to any package comprising a virus particle and a binding molecule presented at the viral surface. Phage-antibodies (pAbs) are likely to find a range of applications in screening antibody V-genes encoding antigen binding activities. For example, pAbs could be used

25 in cloning and rescue of hybridomas (Orlandi, R., et a. (1989) PNAS 86 p3833-3837), and in the screening of large combinatorial libraries (such as found in Huse, W.D. et al., 1989, Science 246, 1275-1281) In particular, rounds of selection using pAbs may help in rescuing the higher

30 affinity antibodies from the latter libraries. It may be preferable to screen small libraries derived from antigen-selected cells (Casali, P., et al., (1986) Science 234 p476-479) to rescue the original VH/VL pairs comprising the V region of an antibody. The use of pAbs may also allow the

35 construction of entirely synthetic antibodies. For example, V-gene repertoires could be made in vitro by combining unrearranged V genes, with D and J segments. Libraries of

pAbs could then be selected by binding to antigen, hypermutated in the antigen-binding loops in vitro and subjected to further rounds of selection and mutagenesis.

The demonstration that a functional antigen-binding site can be expressed on the surface of phage, has implications beyond the construction of novel antibodies. For example, if other protein domains can be expressed at the surface of a phage, phage vectors could be used to clone and select genes by the binding properties of the expressed protein. Furthermore, endless variants of proteins, including epitope libraries built into the surface of the protein, could be made and readily selected for binding activities. In effect other protein architectures might serve as "nouvelle" antibodies. One class of molecules that could be useful in this type of application are receptors. For example, a specific receptor could be expressed on the surface of the phage such that it would bind its ligand. The receptor could then be modified by, for example, in vitro mutagenesis and variants having higher binding affinity for the ligand selected. The screening may be carried out according to one or more of the formats described below with reference to figure 2. Figure 2 refers particularly to phage antibodies. In the situation discussed above the phage antibody is replaced with a phage receptor and the antigen with a ligand 1.

Alternatively, the receptor-phage could be used as the basis of a rapid screening system for the binding of ligands, altered ligands, or potential drug candidates. The advantages of this system of simple cloning, convenient expression, standard reagents and easy handling makes the drug screening application particularly attractive. In the context of this discussion, receptor means a molecule that binds a specific, or group of specific, ligand(s); the natural receptor could be expressed on the surface of a population of cells, it could be the extracellular domain of such a molecule (whether such a form exists naturally or not), or it could be a soluble molecule performing a natural

binding function in the plasma or within a cell or organ.

Although throughout this application the applicant discuss the possibility of screening for higher affinity variants of phage-antibodies, they recognise that in some applications, for example low affinity chromatography (Ohlson, S. et al Anal. Biochem. 169, p204-208 (1988)), it may be desirable to isolate lower affinity variants.

The system of expressing binding molecules on the phage surface could also be used as a primary cloning system. For example, a cDNA library could be constructed and inserted into the bacteriophage and this library screened for the ability to bind a ligand. The ligand/binding molecule combination could include receptor/ligand, enzyme/substrate (or analogue), nucleic acid binding protein/nucleic acid etc. This could be a preferred method to isolate a clone of either molecule in the pair, if the other molecule was available.

Targeted gene transfer

A useful and novel set of applications makes use of the binding protein on the phage to target the phage genome to a particular cell or group of cells. For example, a phage-antibody specific for a cell surface receptor could be used to bind to the target cell surface. The phage could then be internalised, either through the action of the receptor itself or as the result of another event (eg. an electrical discharge such as in the technique of electroporation). The phage genome would then be expressed if the relevent control signals (for transcription and translation and possibly replication) were present. This would be particularly useful if the genome contained a sequence whose expression was desired in the target cell (along with the appropriate expression control sequences). A useful sequence might confer antibiotic resistance to the recipient cell or label the cell by the expression of its product (eg. if the sequence expressed a detectable gene product such as a

luciferase, see White, M, et al, Techniques 2(4), p194-201 (1990)), or confer a particular property on the target cell (eg. if the target cell was a tumour cell and the new sequence directed the expression of a tumour suppressing

5 gene), or express an antisense construct designed to turn off a gene or set of genes in the target cell, or express a gene or gene product designed to be toxic to the target cell.

This technique of "targeted gene transfer" has a number

10 of uses in research and also in therapy and diagnostics. For example, gene therapy often aims to target the replacement gene to a specific cell type that is deficient in its activity; targeted pAbs provide an answer to this problem.   In diagnostics, phage specific for particular

15 bacteria or groups of bacteria have been used to target marker genes, eg. luciferase, to the bacterial host (sec, for example, Ulitzer, S., and Kuhn, J., EPA 85303913.9). If the host range of the phage is appropriate, only those bacteria that are being tested for, will be infected by the

20 phage, express the luciferase gene and be detected by the light they emit. This system has been used to detect the presence of Salmonella.   One major problem with this approach is the initial isolation of a bacteriophage with the correct host range and then cloning a luciferase gene

25 cassette into that phage, such that it is functional. The pAb system allows the luciferase cassette to be cloned into a well characterised system (filamentous phage) and allows simple selection of an appropriate host range, by modifying the antibody (or other binding molecule) specificity the pAb

30 contains.

The applicant has also shown that enzymes can be expressed on the phage surface. Useful applications of this invention include the cloning of enzyme coding genes, or the design and selection of mutant enzymes with enhanced

35 properties on particular substrates.   For example, conditions can be used whereby the enzyme (or modified enzyme) binds a particular substrate, product --

intermediate (or analogues of them) to identify phage from a library containing a desired activity or by subjecting phage already expressing the enzyme, to in vitro mutagenesis, followed by selection of those variants with a desired level

5 of binding and/or catalysis.

The present applicants have also been able to develop novel screening systems and assay formats which depend on the unique properties of these packages eg phage antibodies.

The present invention provides a method for producing a

10 package which method comprises the steps of:

a) inserting a nucleotide sequence encoding the binding molecule within a viral genome;

b) culturing the virus containing said nucleotide sequence, so that said binding molecule is expressed by

15 the virus presented at its surface.

The present invention also provides a method for producing a binding molecule specific for a particular epitope which ·comprises producing a package as describe above and the additional step of screening for said binding

20 molecule by binding of said molecule to said epitope. The method may comprise one or more of the additional steps of: i) separating the package from the epitope; ii) recovering said package; and iii) using the inserted nucleotide sequence in a recombinant system to produce the binding

25 molecule separate from virus. The screening step may isolate the nucleotide sequence encoding the binding molecule of desired specificity, by virtue of said binding molecule being expressed in association with the surface of the virus.

30 In the above methods, the binding molecule may be an antibody, or a fragment or derivative of an antibody. Alternatively, the binding molecule may be an enzyme or receptor and fragments/derivatives of any such enzymes or receptors.

35 In the above methods, the virus may be a filamentous F-specific bacteriophage. The filamentous F-specific bacteriophage may be fd. In particular, it may be a

tetracycline resistant version of fd known as fd-tet. The nucleotide sequence may be inserted within the gene III region of fd. The sequence may be inserted after the signal sequence of gene III, preferably after amino acid+1 of the
5 mature protein. The site for insertion may be flanked by short sequences corresponding to sequences which occur at each end of the DNA to be inserted. For example, the protein domain is an immunoglobulin domain, the insertion site in the phage may be flanked by nucleotide sequences
10 which code for the first five amino acids and the last five amino acids of the Ig domain. Such flanking nucleotide sequences are shown in figure 4(2) B and C, wherein the site-flanking nucleotide sequences encode amino acid sequences QVQLQ and VTVSS which occur at either end of the
15 V_H domain, or QVQLQ and LEIKR which occur at either end of the F_V (combined V_H + V_L) domain. Each of these sequences flanking the insertion site may include a suitable cleavage site, as shown in Fig 4.

Alternatively, the flanking nucleotide sequences shown
20 in figure 4(2) B and C as described above, may be used to flank the insertion site for any DNA to be inserted, whether or not that DNA codes an immunoglobulin.

In the above methods the nucleotide sequences inserted within the viral genome may be derived from eg mammalian
25 spleen cells or peripheral blood lymphocytes. The mammal may be immunised or non-immunised. Alternatively, the nucleotide sequence may be derived by the in vitro mutagenesis of an existing antibody coding sequence. The phage particle presenting said binding molecule may remain
30 intact and infectious.

As previously mentioned, the present invention also provides novel screening systems and assay formats. In these systems and formats the gene sequence encoding the binding molecule (eg the antibody) of desired specificity is
35 separated from the general population having a range of specificities by the fact of its binding to a specific target (eg the antigen or epitope).

Thus, the present invention provides a method of screening a population of phage antibodies (where the binding molecule is an antibody) for a phage antibody with a desired specificity, which comprises contacting said
5   population of phage antibodies with a desired epitope and separating phage antibody which binds to said epitope, from said epitope. The means for separating any binding phage antibodies may be varied in order to obtain binding phage antibodies with different binding affinities for said
10   epitope.

Alternatively, in order to obtain high affinity phage antibodies the epitope may be presented to the population of phage antibodies already with a binding member for said epitope bound thereto, in which case, phage antibodies with
15   a higher binding affinity for said epitope than said bound binding member will displace said bound binding member. The high affinity phage antibodies can then be separated from said epitope.

Separation of phage antibodies from said epitope may be
20   achieved by eg elution techniques well known in the art, infection of suitable bacteria etc.

The present invention also provides packages as defined above and binding molecules (eg antibodies, enzymes, receptors; fragments and derivatives thereof), obtainable by
25   use of any of the above defined methods, systems and formats.

The applicants have chosen the filamentous F- specific bacteriophages as an example of the type of phage that could provide a vehicle for the expression of antibodies and
30   antibody fragments and derivatives on their surface and facilitate subsequent screening and manipulation.

The F-specific phages (eg fl, fd and M13) have evolved a method of propagation which does not kill the host cell and they are used commonly as vehicles for recombinant DNA
35   (Kornberg, A., DNA Replication, W.H. Freeman and Co., San Francisco, 1980). The single stranded DNA genome (approximately 6.4 Kb) is extruded through the bacterial

membrane where it sequesters capsid sub-units, to produce mature virions. These virions are 6 nm in diameter, 1μm in length and each contain approximately 2,800 molecules of the major coat protein encoded by viral gene VIII and four

5 molecules of the adsorption molecule encoded by viral gene III. The latter is located at one end of the virion. The structure has been reviewed by Webster et al., 1978 in The Single Stranded DNA Phages, 557-569, Cold Spring Harbor Laboratory Press. The gene III product is involved in the

10 binding of the phage to the bacterial F-pilus.

Although these phages do not kill their host during normal replication, disruption of some of their genes can lead to cell death (Kornberg, A., 1980 supra.) This places some restraint on their use. The applicants have recognized

15 that gene III of phage fd is an attractive possibility for the insertion of biologically active foreign sequences. The protein itself is only a minor component of the phage coat and disruption of the gene does not lead to cell death (Smith, G. 1988, Virology 167: 156-165). Furthermore, it is

20 possible to insert some foreign sequences (with no biological function) into various positions within this gene (Smith, G., 1985 Science 228: 1315-1317., Parmley, S.F. and Smith, G.P Gene: 73 (1988) p. 305-318., and de la Cruz, V.F., et al., 1988, J. Biol. Chem., 263: 4318-4322). In

25 these cases, although the infectivity of the virion was disrupted, the inserted sequences could be detected on the phage surface.

The protein encoded by gene III has several domains (Pratt, D., et al., 1969 Virology 39:42-53., Grant, R.A., et

30 al., 1981, J. Biol. Chem. 256: 539-546 and Armstrong, J., et al., FEBS Lett. 135: 167-172 1981.) including: i) a signal sequence that directs the protein to the cell membrane and which is then cleaved off; ii) a domain that anchors the mature protein into the bacterial cell membrane (and also

35 the phage coat); and iii) a domain that specifically binds to the phage receptor the F-pilus of the host bacterium. Short sequences derived from protein molecules have been

inserted into two places within the mature molecule (Smith. G., 1985 supra., and Parmley, S.F. and Smith, G.P., 1988 supra.) into an inter-domain region and also between amino acids 2 and 3 at the N-terminus. The insertion sites at the

5   N-terminus were more successful in maintaining the structural integrity of the gene III protein and displaying the peptides on the surface of the phage. By use of specific antisera, the peptides inserted into this position were shown to be on the surface of the phage. These authors

10   were also able to purify the phage using this property. However, the peptides expressed by the phage, did not possess measurable biological functions of their own.

Retaining the biological function of a molecule when it is expressed in a radically different context to its natural

15   state is difficult. The demands on the structure of the molecule are heavy. In contrast, retaining the ability to be bound by specific antisera is a passive process which imposes far less rigorous demands on the structure of the molecule. For example, it is the rule rather than the

20   exception that polyclonal antisera will recognise totally denatured, and biologically inactive, proteins on Western blots (see for example, Harlow, E. and Lane, D., Antibodies, a Laboratory Manual, Cold Spring Harbor Laboratory Press 1988). Therefore, the insertion of peptides into a regic

25   that allows their structure to be probed with antisera teaches only that the region allows the inserted sequences to be exposed and does not teach that the region is suitable for the insertion of large sequences with demanding biological function.

30   This experience with Western blots is a graphic practical demonstration which shows that retaining the ability to be bound by specific antisera imposes far less rigorous demands on the structure of a molecule, than does the retention of a biological function.

35   The applicants have investigated the possibility of inserting biologically active antibody fragments into the gene III region of fd to create a large fusion protein. As

is apparent from the previous discussion, this approach makes onerous demands on the functionality of the fusion protein. The insertion is large, 100-200 amino acids; the antibody derived domain must fold efficiently and correctly to retain antigen-binding; and most of the functions of gene III must be retained. The applicants approach to the construction of the fusion molecule was designed to minimise the risk of disrupting these functions. The initial vector used was fd-tet (Zacher, A.N., et al., 1980, Gene 9, 127-140) a tetracycline resistant version of fd bacteriophage that can be propagated as a plasmid that confers tetracycline resistance to the infected E.coli host. The applicants chose to insert after the signal sequence of the fd gene III protein for several reasons. In particular, the applicants chose to insert after amino acid 1 of the mature protein to retain the context for the signal peptidase cleavage. To retain the structure and function of gene III itself, the majority of the original amino acids are synthesized after the inserted immunoglobulin sequences. The inserted immunoglobulin sequences were designed to include residues from the switch region that links V_H-V_L to C_H1-C_L (Lesk, A., and Chothia, C., Nature 335, 188-190, 1988).

Surprisingly, by manipulating gene III of bacteriophage fd, the present applicants have been able to construct a bacteriophage that expresses on its surface large biologically functional antibody molecules and which remains intact and infectious. Furthermore, the phages bearing antibodies of the correct specificity, can be selected from a background where the majority of phages do no show this specificity.

The population of antibody molecules inserted into the phage can be derived from a variety of sources. For example, immunised or non-immunised rodents or humans, and from organs such as spleen and peripheral blood lymphocytes. The coding sequences are derived from these sources by techniques familiar to those skilled in the art (Orland .,

R., et al., 1989 supra; Larrick, J.W., et al., 1989 supra; Chiang, Y.L., et al., 1989 Bio Techniques 7, p. 360-366; Ward, E.S, et al., 1989 supra; Sastry, L., et al., 1989 supra.) Each individual phage antibody in the

5   resulting library of phage antibodies will express antibody derived fragments that are monoclonal with respect to its antigen-binding characteristics.

The disclosure made by the present applicants is important and provides a significant breakthrough in the

10   technology relating to the production of biological binding molecules, their fragments and derivatives by the use of recombinant methods.

In standard recombinant techniques for the production of antibodies, an expression vector containing sequences

15   coding for the antibody polypeptide chains is used to transform eg E.coli. The antibody polypeptides are expressed and detected by use of standard screening systems. When the screen detects an antibody polypeptide of the desired specificity, one has to return to the particular

20   transformed E.coli expressing the desired antibody polypeptide. Furthermore, the vector containing the coding sequence for the desired antibody polypeptide then has to be isolated for use from E.coli in the further processing steps.

25   In the present invention however, the desired antibody polypeptide when expressed, is already packaged with its gene coding sequence. This means that when the screen detects an antibody polypeptide of desired specificity, there is no need to return to the original culture for

30   isolation of that sequence.

Because the phage antibody is a novel structure that contains an antibody of monoclonal antigen-binding specificity on the surface of a relatively simple structure also containing the genetic information encoding its

35   function, phage antibodies that bind antigen can be recovered very efficiently by either eluting off (eg using diethylamine, high salt etc) and infecting suitab'...

bacterial or by denaturing the structure and specifically amplifying the antibody encoding sequences using PCR. That is, there is no necessity to refer back to the original bacterial clone that gave rise to the phage antibody.

5   Individual phage antibodies expressing the desired antigen-binding specificity can be isolated from the complex library using the conventional screening techniques (eg as described in Harlow, E., and Lane, D., 1988, supra). One example is illustrated in figure 2(i). This shows antigen

10 (ag) bound to a solid surface (s). The population of phage antibodies is then passed over the antigen, and those individuals p that bind are retained after washing, and optionally detected with detection system d. One possible detection system based upon anti-fd antisera is illustrated

15 below in example 4. Since the bound phage antibody can be amplified using for example PCR or bacterial infection, it is also possible to rescue the desired specificity even when insufficient individuals are bound to allow detection via conventional techniques.

20 The efficiency of this screening procedure for phage antibodies and the ability to create very large libraries means that the immunisation techniques developed to increase the proportion of screened cells producing antibodies of interest will not be an absolute requirement. The technique

25 allows the rapid isolation of antigen-binding specificities, including those that would be difficult or even unobtainable by conventional techniques, for example, catalytic or anti-idiotypic antibodies. Removal of the animal altogether is now possible once a complete library of the immune

30 repertoire has been constructed.

Affinity Maturation Screening Formats

The applicants have also devised a series of novel screening techniques that are practicable only because of the unique properties of phage antibodies. The general

35 outline of some screening procedures is illustrated in figure 2.

The population/library of phage antibodies to be

screened could be generated from immunised or other animals; or be created in vitro by mutagenising pre-existing phageantibodies (using techniques well-known in the art such as oligonucleotide directed mutagenesis (Sambrook, J., et al., 1989 Molecular Cloning a Laboratory Manual, Cold Spring Harbor Laboratory Press). This population can be screened in one or more of the formats described below with reference to figure 2, to derive those individual phage antibodies whose antigen binding properties are different from sample c. Examples of the possible screening formats are:

Binding/Elution

Referring to figure 2(i) population p binds to antigen ag fixed to a solid support s. If samples of bound population p are removed under increasingly stringent conditions, the binding affinity represented in each sample will increase. Conditions of increased stringency can be obtained, for example, by increasing the time of soaking or changing the pH of the soak solution, etc.

Competition

Referring to figure 2(ii) antigen ag can be bound to a solid support s and bound to saturation by the original binding molecule c. If a population of mutant phage antibody (or a set of unrelated phage antibody) p is offered to the complex, only those that have higher affinity for antigen ag than c will bind. In most examples, only a minority of population c will be displaced by individuals from population p. If c is a traditional antibody molecule, all bound material can be recovered and bound p recovered by infecting suitable bacteria and/or by use of standard techniques such as PCR.

An advantageous application is where ag is used as a receptor and c the corresponding ligand. The recovered bound population p is then related structurally to the receptor binding site/and or ligand. This type of specificity is known to be very useful in the pharmaceutical

industry.

Another advantageous application is where ag is an antibody and c its antigen. The recovered bound population p is then an anti-idiotype antibody which have numerous uses in research and the diagnostic and pharmaceutical industries.

In some instances it may prove advantageous to pre-select population p. For example, in the anti-idiotype example above, p can be absorbed against a related antibody that does not bind the antigen.

However, if c is a phage antibody, then either or both c and p can advantageously be marked in some way to both distinguish and select for bound p over bound c. This marking can be physical, for example, by pre-labelling p with biotin; or more advantageously, genetic. For example, c can be marked with an EcoB restriction site, whilst p can be marked with an EcoK restriction site (see Carter, P. et al., 1985, Nucl. Acids Res. 13, 4431-4443). When bound p+c are eluted from the antigen and used to infect bacteria, there is restriction (and thus no growth) of population c (i.e. EcoB restricting bacteria in this example). Any phage that grew, would be greatly enriched for those individuals from p with higher binding affinities. Alternatively, the genetic marking can be achieved by marking p with new sequences, which can be used to specifically amplify p from the mixture using PCR.

The novel structure of the phage antibody molecule can be used in a number of other applications some examples of which are:

Signal Amplification

Acting as a novel molecular entity in itself, phage antibodies combine the ability to bind the specific antigen with the amplification, if the major coat protein is used to attach another moiety. This moiety can be attached via immunological, chemical, or any other means and can be used,

for example, to label the complex with detection reagents or cytotoxic molecules for use in vivo or in vitro.

Physical Detection

The size of the phage antibody can be used as a marker particularly with respect to physical methods of detection such as electron microscopy and/or some biosensors, eg. surface plasmon resonance.

Diagnostic Assays

The phage antibody molecule also has advantageous uses in diagnostic assays, particularly where separation can be effected using its physical properties for example centrifugation, filtration etc.

In order that the invention is more fully understood, embodiments will now be described in more detail by way of example only and not by way of limitation with reference to the figures described below.

Figure 1 shows the basic structure of the simplest antibody molecule IgG.

Figure 2 shows schematically novel screening techniques which utilise the unique properties of phage antibodies.

Figure 3 shows a scheme for the construction of vectors.

Figure 4 shows the nucleotide sequences for the oligonucleotides and vectors. All sequences are drawn 5' to 3' and are numbered according to Beck et al., 1978, Nucl. Acid Res., 5: 4495-4503. 4.1 shows the sequences of the ologonucleotides used for mutagenesis (oligo's 1 and 2) or sequencing (oligo 3). The sequences shown were synthesized on an Applied Biosystems, olgonucleotide synthesizer and are complementary to the single stranded form of fd-tet (they are in the anti-sense form with respect to gene III). 4.2 shows the sequences of the various constructs around the gene III insertion site. these sequences are drawn in the sense orientation with respect to gene III; a) fd-tet (and

FDTdBst) b) FDTPs/Bs and c) FDTPs/Xh. The key restriction enzyme sites are shown along with the immunoglobulin amino acids contributed by the vectors, (amino acid single letter code is used, see Harlow, E., and Lane, D., 1988 supra.).

Figure 5 shows the nucleotide and amino acid sequences for scFv in the vector scFvD1.3 myc. This gives the sequence of the anti-lysozyme single chain Fv and surrounding sequences in scFvD1.3 myc showing the N-terminal pel B signal peptide sequence and the C-terminal myc tag sequence (Ward, E.S., et al., 1989, supra.). Also highlighted is the peptide sequence linking the V_H and V_L regions. The amino acid sequence is represented above the nucleotide sequence by the single letter code, see Harlow, E., and Lane, D., 1988 supra..

Figure 6 shows the effect of varying the amount of supernatant on the binding of phage antibodies to lysozyme in graphical form. Each point is the average of duplicate samples. Lysozyme was coated at 1 mg/ml in 50 mM $NaHCO_3$.

Figure 7 shows the effect of varying the coating concentration on the binding of phage antibodies to lysozyme in graphical form. Each point is the average of duplicate samples. Lysozyme was coated with the specified concentration of either BSA or lysozyme.

Figure 8 shows the sequence around the cloning site in gene III of fd-Cat2.

Figure 9 shows the binding of pAb (D1.3) to lysozymes. Binding of phage as detected by ELISA to (a) hen egg-white lysozyme (HEL) (b) turkey egg-white lysozyme (TEL), (c) human lysozyme (HUL), (d) bovine serum albumin (BSA). A further control of (c) fd-CAT1 to HEL.

Figure 10 shows a map of fab D1.3 in pUC19.

Figure 11 shows the ELISA results providing a comparison of lysozyme-binding by phage-Fab and phage-ScFv. Vector=fd-CAT2 (example 5); fdSCFV(OX)=pAbNQ11 (Example 9); fdVHCH1(D1.3)=grown in normal cells (ie. no light chain, see

example 7); fdfab-fdfab-fdVHCH1 (D1.3) grown in cells containing D1.3 light chain; fdSCFV (D1.3)=pAbD1.3.

Figure 12 shows oligonucleotide probing of affinity purified phage. 10^{12} phage in the ratio 1 pAb (D1.3) in

5   4×10^4 fd-CAT1 phages were affinity purified and probed with an oligonucleotide specific for pAb (D1.3) (a) filter after one round of affinity purification (900 colonies total) and, (b) after two rounds (372 colonies total).

10   Figure 13 shows the sequence of the anti-oxazalone antibody NQ11.

Figure 14 shows the ELISA results for binding of pAb NQ11 and pAb D1.3 and Vector FDTPs/xh to specified antigens.

15   Figure 15 shows the sequence surrounding phoA insertion in fd-phoA1. The restriction sites used for cloning are shown, as well as the amino acids encoded by phoA around the insertion site. The first five amino acids of the mature fusion come from gene 3.

20   Figure 16(1) shows the structure of gene 3 and figure 16(2) shows the peptide linker sites A and B.

Figure 17 shows schematically the protocol for PCR assembly of mouse VH and VLK repertoires for phage display described in example 16.

25   Figure 18 shows examples of the final products obtained with the procedure of example 16. Lanes a and b

show the products of the initial PCR using heavy and light chain primers respectively; lane c shows the complete assembled 700bp product before final digestion with Notl and Apall; M1, M2 markers Φ174 Hae III digest

5   and 123 base pair ladder (BRL Limited, P.O. Box 35, Washington Road, Paisley, Scotland) respectively.

Figure 19 shows the binding of ^{125}I-PDGF-BB to fd h-PDGFB-R phage in immunoprecipitation assay and comparison to fd TPs/Bs and no phage controls; binding is expressed

10   as a percentage of the total ^{125}I-PDGF-BB added to the incubation·

Figure 20 shows the ~~displacement~~ *competitive reduction* of ^{125}I-PDGF-BB bound to fd-h-PDGFB-R phage using unlabelled PDGF-BB measured using an immunoprecipitation assay. Binding is

15   expressed as a percentage of the total ^{125}I-PDGF-BB added to the incubation·

Figure 21 shows the ~~displacement~~ *competitive reduction* of ^{125}I-PDGF-BB bound to fd-h-PDGFB-R phage using unlabelled PDGF-BB measured using an immunoprecipitation assay. Non-

20   specific binding of ^{125}I-PDGF-BB to vector phage fdTPs/Bs in the absence of added unlabelled PDGF was deducted from each point.

Figure 22 shows the results of an ELISA of lysozyme binding by pCAT-3 ScFv D1.3 phagemid in comparison with

25   pCAT-3 vector (both rescued by M13KO7) and fdCAT2 ScFv D1.3 as described in example 17. The ELISA was performed

as described in example 6 with modifications detailed in example 17.

The following procedures used by the present applicants are described in Sambrook, J. et al., 1989,

5   supra.: restriction digestion, ligation, preparation of competent cells (Hanahan method), transformation, analysis of restriction enzyme digestion products on agarose gels, purification of DNA using phenol/chloroform, 5'-end labelling of oligonucleotides,

10   filter screening of bacterial colonies, preparation of 2xTY medium and plates, preparation of tetracycline stock solutions, PAGE of proteins, preparation of phosphate buffered saline.

All enzymes were supplied by New England Biolabs (CP

15   Laboratories, PO Box 22, Bishop's Stortford, Herts., England) and were used according to manufacturer's instructions unless otherwise stated.

The vector fd-tet (Zacher, A.N. et al., 1980, supra) was obtained from the American Type Culture Collection

20   (ATCC

25

No. 37000) and transformed into competent TG1 cells (genotype: K12δ (lac-pro), sup E, thi, hsdD5/F'traD36, pro A+B+, Lac 1^q, lac δM15).

Viral particles were prepared by growing TG1 cells containing the desired construct in 10 to 100 mls 2xTY medium with 15 µg/ml tetracycline for 16-24 hours. The culture supernatant was collected by centrifugation for 10 mins at 10,000 rpm in an 8 x 50 ml rotor, Soryal RC-5B centrifuge. Phage particles were precipitated by adding 1/5th volume 20% polyethylene glycol (PEG)/2.5M NaCl and leaving at 4°C for 1 hour. These were spun for 15 minutes as described above and the pellets resuspended in 10 mM Tris/HCI pH 8, 1 mM EDTA to 1/100th of the original volume. Residual bacteria and undissolved material were removed by spinning for 2 minutes in a microcentrifuge. Single stranded DNA for mutagenesis or sequencing was prepared from concentrated phage according to Sambrook, J., et al., 1989, supra.

Example 1

Design of Insertion Point Linkers and Construction of Vectors

The vector fd-tet has two BstEII restriction sites flanking the tetracycline resistance gene (fig 3). Since the strategy for inserting the V_H fragments was to ligate them into a newly inserted BstEII site within gene III, it was advantageous to delete the original BstEII sites from fd-tet. This was achieved by digesting fd-tet with the restriction enzyme BstEII, filling-in the 5' overhangs and re-ligating to generate the vector FDTδBst. Digestion of fd-tet with BstEII (0.5 units/µl) was carried out in 1x KGB buffer (100 mM potassium glutamate, 25 mM Tris-acetate (PH 7.5), 10 mM magnesium acetate, 50 µg/ml bovine serum albumin, 0.5 mM (Sambrook, J., et al., 1989, supra.) with DNA at a concentration of 25 ng/µl. The 5' overhang was filled in, using 2x KGB buffer, 250 µM each dNTP's

(Pharmacia Ltd., Pharmacia House, Midsummer Boulevard, Milton Keynes, Bucks., UK.) and Klenow Fragment (Amersham International, Lincoln Place, Green End, Aylesbury, Bucks., UK) at 0.04 units/ µl. After incubating for 1 hour at room temperature, DNA was extracted with phenol/chloroform and precipitated with ethanol.

Ligations were carried out at a DNA concentration of 50ng/µl for 1 hour at room temperature using T4 DNA ligase (40 units/µl). Ligations were transformed into competent TG1 cells and plated onto TY plates supplemented with 15 µg/ml tetracycline. Colonies were picked into 25 mls of 2xTY medium supplemented with 15 µg/ml tetracycline and grown overnight at 37°C.

Double stranded DNA was purified from the resulting clones using the gene-clean II kit (Bio101 Inc., PO Box 2284, La Jolla, California, 92038-2284, USA.) and according to the small scale rapid plasmid DNA isolation procedure described therein. The orientation of 5 of the resulting clones was checked using the restriction enzyme ClaI. A clone was chosen which gave the same ClaI pattern as fd-tet, but which had no BstE II sites.

In vitro mutagenesis of FDT6Bst was used to generate vectors that facilitated cloning of antibody fragments downstream of the gene III signal peptide and in frame with the gene III coding sequence. The oligonucleotide directed mutagenesis system, version 2 (Amersham International) was used with oligo 1 (figure 4) to create FDTPs/Bs. The sequence of FDTPs/Bs (figure 4) was confirmed using the sequenase version 2.0 kit (USB Corp., PO Box 22400, Cleveland, Ohio, 44122, USA.) with oligo 3 (figure 4) as a primer.

A second vector FDTPs/Xh (to facilitate cloning of single chain F_V fragments) was generated by mutagenising FDTPs/Bs with oligo 2 according to the method of Venkitaraman, A.R., Nucl. Acid Res. 17, p 3314. The

sequence of FDTPs/Xh (figure 4) was confirmed using the sequenase version 2.0 kit (USB Corp.) with oligo 3 as a primer.

Clearly, alternative constructions will be apparent to those skilled in the art. For example, M13 and/or its host bacteria could be modified such that its gene III could be disrupted without the onset of excessive cell death; the modified fd gene III, or other modified protein, could be incorporated into a plasmid containing a single stranded phage replication origin, such as pUC119, superinfection with modified phage such as KO7 would then result in the encapsulation of the phage antibody genome in a coat partially derived from the helper phage and partly from the phage antibody gene III construct.

The detailed construction of a vector such as FDTPs/Bs is only one way of achieving the end of a phage antibody. For example, techniques such as sticky feet cloning/mutagenesis (Clackson, T. and Winter, G. 1989 Nucl. Acids. Res., 17, p 10163-10170) could be used to avoid use of restriction enzyme digests and/or ligation steps.

Example 2.

Insertion of Immunoglobulin F_V Domain into Phage Antibody

The plasmid scFv D1.3 myc (gift from G. Winter and A. Griffiths) contains V_H and V_L sequences from the antibody D1.3 fused via a peptide linker sequence to form a single chain F_V version of antibody D1.3. The sequence of the scF$_v$ and surrounding sequences in scFvD1.3 myc is shown in figure 5.

The D1.3 antibody is directed against hen egg lysozyme (Harper, M. et al., 1987, Molec. Immunol. 24, 97-108) and the scFv form expressed in E. coli has the same specificity (A. Griffiths and G. Winter personal Communication).

Digestion of scFv D1.3 myc with PstI and XhoI, excises a fragment of 693 bp which encodes the bulk of the scFv. Ligation of this fragment into FDTPs/Xh cleaved with PstI

and XhoI gave rise to the construct FDTSCFVD1.3 encoding the gene III signal peptide and first amino acid fused to the complete D1.3 scFv, followed by the mature gene III protein from amino acid 2.

The vector FDTPs/Xh was prepared for ligation by digesting with the PstI and XhoI for 2 hours followed by digestion with calf intestinal alkaline phosphatase (Boehringer Mannheim UK Ltd., Bell Lane, Lewes, East Sussex, BN7 1LG) at one unit/ul for 30 minutes at 37°C. Fresh calf intestinal alkaline phosphatase was added to a final total concentration of 2 units/ul and incubated for a further 30 minutes at 37°C. The reaction was extracted three times with phenol/chloroform, precipitated with ethanol and dissolved in water. The insert from scFvD1.3 myc was excised with the appropriate restriction enzymes, extracted twice with phenol/chloroform, precipitated with ethanol and dissolved in water. Ligations were carried out as described in example 1 except both vector and insert samples were at a final concentration of 5 ng/ul each. The formation of the correct construct was confirmed by sequencing as described in example 1.

To demonstrate that proteins of the expected size were produced, virions were concentrated by PEG precipitation as described above, and the equivalent of 2mls of supernatant was loaded onto an 18% SDS polyacrylamide gel. After electrophoresis, the gel was soaked in gel running buffer (50 mM Tris, 380 mM Glycine, 0.1%SDS) with 20% methanol for 15 minutes. Transfer to nitrocellulose filter was executed in fresh 1x running buffer/20% methanol using TE70 Semi Phor a semi-dry blotting apparatus (Hoeffer, 654 Minnesota Street, Box 77387, San Francisco, California 94107, USA.).

After transfer, the filter was blocked by incubation for 1 hour in a 2% solution of milk powder (Cadbury's Marvel) in phosphate buffered saline (PBS). Detection of F_V and V_H protein sequences in the phage antibody fusion proteins was effected by soaking the filter for 1 hour with a 1/1000 dilution (in 2% milk powder) of a rabbit polyclonal

antiserum raised against affinity purified, bacterially expressed F_V fragment (gift from G. Winter). After washing PBS (3 x 5 minute washes), bound primary antibody was detected using an anti-rabbit antibody conjugated to horseradish peroxidase (Sigma, Fancy Road, Poole, Dorset, BH17 7NH, UK.) for 1 hour. The filter was washed in PBS/0.1% triton X-100 and developed with 0.5 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (DAB), 0.02% cobalt chloride, 0.03% hydrogen peroxide in PBS.

The results show that with FDTVHD1.3 (from example 3) and FDTSCVFVD1.3, a protein of between 69,000 and 92,500 daltons is detected by the anti-F_V serum. This is the expected size for the fusion proteins constructed. This product is not observed in supernatants derived from fd-tet, FDTδBst or FDTPs/Xh.

Example 3.

Insertion of Immunoglobulin V_H Domain into Phage Antibody

The VH fragment from D1.3 was generated from the plasmid pSW1-VHD1.3-TAG1 (Ward, E.S. et al., 1989 supra.). Digestion with Pst1 and BstEII generates the fragment shown between positions 113 and 432 in figure 5. Cloning of this fragment into the Pst1 and BstEII sites of FDTPs/Bs gave rise to the construct FDTVHD1.3 which encodes a fusion protein with a complete V_H inserted between the first and third amino acids of the mature gene III protein (amino acid two has been deleted).

The methods used were exactly as in example 2 except that the vector used was FDTPs/Bs digested with Pst1 and BstEII.

Example 4.

Analysis of Binding Specificity of Phage Antibodies

The binding of the various phage antibodies to the specific antigen, lysozyme, was analysed using ELISA techniques. Phage antibodies were grown in E.coli and phage antibody particles were precipitated with PEG as in the materials and methods. Bound phage antibody particles were detected using polyclonal rabbit serum raised against the

closely related phage M13.

ELISA plates were prepared by coating 96 well plates (Falcon Microtest III flexible plate. Falcon: Becton Dickinson Labware, 1950 Williams Drive, Oxnard, Californa, 93030, USA.) with 200 ul of a solution of lysozyme (1mg/ml unless otherwise stated) in 50 mm $NaHCO_3$ for 16-24 hours. Before use, this solution was removed, the plate rinsed several times in PBS and incubated with 200 ul of 2% milk powder/PBS for 1 hour. After rinsing several times with PBS, 100 ul of the test samples were added and incubated for 1 hour. Plates were washed (3 rinses in 0.05% Tween 20/PBS followed by 3 rinses in PBS alone). Bound phage antibodies were detected by adding 200 ul/well of a 1/1000 dilution of sheep anti-M13 polyclonal antiserum (gift from G. Winter) in 2% milk powder/PBS and incubating for 1 hour. After washing as above, plates were incubated with biotinylated anti-sheep antibody (Amersham International) for 30 minutes. Plate were washed as above, and incubated with streptavidin-horseradish peroxidase complex (Amersham International). After a final wash as above, 0.5 mg/ml ABTS substrate in citrate buffer was added (ABTS = 2'2'-azinobis(3-ethylbenzthiazoline sulphonic acid); citrate buffer =50 mM citric acid, 50 mM tri-sodium citrate at a ratio of 54:46). Hydrogen peroxide was added to a final concentration o. 0.003% and the plates incubated for 1 hour. The optical density at 405 nm was read in a Titertek multiskan plate reader.

Figure 6 shows the effect of varying the amount of phage antibody. 100 ul of various dilutions of PEG precipitated phage were applied and the amount expressed in terms of the original culture volume from which it was derived. Signals derived from both the scFv containing phage antibody (FDTSCFVD1.3) and the V_H containing phage antibody (FDTVHD1.3) were higher than that derived from the phage antibody vector (FDTPs/Xh). The highest signal to noise ratio occurs using the equivalent of 1.3 mls of culture.

Figure 7 shows the results of coating the plates with varying concentrations of lysozyme or bovine serum albumin (BSA). The equivalent of 1 ml of the original phage antibody culture supernatant was used. The signals from supernatants derived from FDTSCFVD1.3 were again higher than those derived from FDTPs/Xh when lysozyme coated wells were used. There was no significant difference between these two types of supernatant when the plates were coated with BSA. Broadly speaking the level of signal on the plates is proportional to the amount of lysozyme coated. These results demonstrate that the binding detected is specific for lysozyme as the antigen.

Example 5.

Construction of fd Cat 2

It would be useful to design vectors that enable the use of restriction enzymes that cut DNA infrequently, thus avoiding unwanted digestion of the antibody gene inserts within their coding sequence. Enzymes with an eight base recognition sequence are particularly useful in this respect, for example Notl and Sfil. Chaudhary et al (PNAS 87 p1066-1070, 1990) have identified a number of restriction sites which occur rarely in antibody variable genes. The applicant has designed and constructed a vector that utilises two of these sites, as an example of how this type of enzyme can be used. Essentially sites for the enzymes ApaLl and Notl were engineered into FDTPs/Xh to create fdCAT2.

The oligonucleotide:

5'ACT TTC AAC AGT TTC TGC GGC CGC CCG TTT GAT CTC GAG CTC CTG CAG TTG GAC CTG TGC ACT GTG AGA ATA GAA 3'

was synthesised (supra fig 4 legend) and used to mutagenise FDTPs/Xh using an in vitro mutagenesis kit from Amersham International as described in example 1, to create fd-CAT2. The sequence of fd-CAT2 was checked around the site of manipulation by DNA sequencing. The final sequence around the insertion point within gene III is shown in figure 8.

Example 6

<u>Specific Binding of Phage-antibody(pAb) to Antigen</u>

The binding of pAb D1.3 (FDTSCFVD1.3 of example 2) to lysozyme was further analysed by ELISA.

<u>Methods.</u>

<u>1. Phage growth.</u>

Cultures of phage transduced bacteria were prepared in 10-100 mls 2 x YT medium with 15 µg/ml tetracycline and grown with shaking at 37°C for 16-24 hrs. Phage supernatant was prepared by centrifugation of the culture (10 min at 10,000 rpm, 8 x 50 ml rotor, Sorval RC-5B centrifuge). At this stage, the phage titre was $1 - 5 \times 10^{10}$ ml^{-1} transducing units. The phage were precipitated by adding 1/5 volume 20% polyethylene glycol, 2.5 M NaCl, leaving for 1 hr at 4°C, and centrifuging (supra). The phage pellets were resuspended in 10 mM Tris-HCl, 1mM EDTA pH 8.0 to 1/100th of the original volume, and residual bacteria and aggregated phage removed by centrifugation for 2 min in a benc microcentrifuge.

<u>ELISA</u>

Plates were coated with antigen (1 mg ml^{-1} antigen) and blocked as described in example 4. 2×10^{10} phage transducing units were added to the antigen coated plates in phosphate buffered saline (PBS) containing 2% skimmed milk powder (MPBS). Plates were washed between each step wit three rinses of 0.5% Tween-20 in PBS followed by three rinses of PBS. Bound phage was developed by incubating with sheep anti-M13 antisera and detected with horseradish peroxidase (HRP) conjugated anti-goat serum (Sigma, Poole, Dorset, UK) and ABTS (2'2'-azinobis (3-ethylbenzthiazoline sulphonic acid). Readings were taken at 405 nm after a suitable period. The results (figure 9) show that the antibody bearing-phage had the same pattern of reactivity as the original D1.3 antibody (Harper, M., Lema, F., Boulot, G., and Poljak, F.J. (1987) Molec. Immunol. 24, 97-108), and bound to hen egg-white lysozyme, but not to turkey egg-white lysozyme, human lysozyme or bovine serum albumin. The specificity of the phage is particularly illustrated by the

lack of binding to the turkey egg-white lysozyme that differs from hen egg-white lysozyme by only 7 amino acids.

Example 7

Expression of Fab D1.3 in fd CAT2

The aim of this example was to demonstrate that the scFv format used in example 2 was only one way of displaying antibody fragments in the pAb system. A more commonly used antibody fragment is the Fab fragment (figure 1) this example describes the construction of a pAb that expresses a Fab-like fragment on its surface and shows that it binds specifically to its antigen. The applicant chose to express the heavy chain of the antibody fragment consisting of the VH1 and CH1 domains in the pAb itself and to co-express the light chain in the same cell; the VH and CH1 regions of anti-lysozyme antibody D1.3 were cloned in fd CAT2, and the corresponding light chain cloned in plasmid pUC19. The light chain associates with the heavy chain-gene III fusion, as it does when the heavy and light chains are expressed as independant proteins (Skerra, A and Pluckthun, A. Science 240, p1038-1040 (1988)).

It is possible to express the light chain from within the pAb genome by, for example, cloning an expression cassette into a suitable place in the phage genome. Such a suitable place would be the intergenic region which houses the multicloning sites engineered into derivative of the related phage M13 (see, for example, Yanisch-Perron, C. et al., Gene 33, p103-119, (1985)).

The starting point for this example was the clone Fab D1.3 in pUC19, a map of which is shown in figure 10. The sequence encoding the VH-CH1 region was PCR amplified from Fab D1.3 in pUC19 using oligonucleotides KSJ 6 and 7, which retain the Pst I site at the 5'end and introduce a Xho I site at the 3'end, to facilitate cloning into fd CAT2. The sequences for the oligonucleotides KSJ 6 and 7 are shown below.

KSJ6: 5' AGG TGC AGC TGC AGG AGT CAG G 3'

KSJ7: 5' GGT GAC CTC GAG TGA AGA TTT GGG CTC AAC TTT C 3'

PCR conditions were as described in example II, except that thirty cycles of PCR amplification were performed with denaturation at 92°C for 45 seconds, annealing at 55°C for 1 minute and extension at 72°C for 1 minute. The template used was TG1 cells containing Fab D1.3 in pUC19 resuspended in water and boiled (see example 12). This regime resulted in amplification of the expected fragment of approximately 600bp. This fragment was cut with Pst I and Xho I, purified from an agarose gel and ligated into Pst 1/Xho 1-cut fd CAT2 as described in example 12. Part of the ligation was transformed into E.coli MC1061 (Available from, for example, Clontech Laboratories Inc, Palo Alto, California) and colonies identified by hybridisation with the oligonucleotide D1.3CDR3A as described in example 10. The presence of the VHCH1 gene fragment was likewise confirmed by PCR, using oligonucleotides KSJ6 and 7. A representative clone was called fd CAT2: VHCH1 D1.3.

The heavy chain was deleted from fab D1.3 in pUC19 by Sph I cleavage of fab D1.3 plasmid DNA. The 2.7Kb fragment containing pUC19 and the light chain gene was purified from a TAE agarose gel, and 10ng of this DNA self-ligated and transformed into competent E.coli TG1. Cells were plated on 2YT agar containing ampicillin (100μg/ml) and incubated at 30°C overnight. The resulting colonies were used to mak miniprep DNA (Sambrook et al. supra), and the absence of the heavy chain gene confirmed by digestion with Sph I and Hind III. A representative clone was called LC D1.3 DHC.

An overnight culture of fd CAT2; VHCH1 D1.3 cells was microcentrifuged at 13,000Xg for 10 minutes and 50μl of the supernatant containing phage particles added to 50μl of an overnight culture of LC D1.3 DHC cells. The cells were incubated at 37°C for 10 minutes and plated on 2YT agar containing ampicillin (100μg/ml) and 15mg/ml tetracycline. Phage were prepared from some of the resulting colonies and assayed for their ability to bind lysozyme as described in example 6.

The results (Figure 11) showed that when the heavy and

light chain Fab derivatives from the original anitbody Dl.3
were present, the pAb bound to lysozyme. pAb expressing the
fd VHCH1 fragment did not bind to lysozyme unless grown in
cells also expressing the light chain. This shows that a
functional Fab fragment was produced by an association of
the free light chain with the VHCH1 fragment fused to gene
III and expressed on the surface of the pAb.

Example 8

Isolation of Specific, Desired Phage from a Mixture of
Vector Phage.

The applicant purified pAb (Dl.3) (originally called
FDTSCFVDl.3 in example 2) from mixtures using antigen
affinity columns. pAb (Dl.3) was mixed with vector fd phage
(see table 1) and approximately 10^{12} phage passed over a
column of lysozyme-Sepharose (prepared from cyanogen bromide
activated sepharose 4B (Pharmacia, Milton Keynes, Bucks,
UK.) according to the manufacturers instructions. TG1 cells
were infected with appropriate dilutions of the elutes and
colonies derived, were analysed by probing with an
oligonucleotide that detects only the pAb (Dl.3) (see Table
1 and Fig. 12) A thousand fold enrichment of pAb (Dl.3) was
seen with a single column pass. By growing the enriched
phage and passing it down the column again, enrichments of
up to a million fold were seen.

Enrichment was also demonstrated using purely
immunological criteria. For example, 10^{12} phage (at a ratio
of 1 pAb (Dl.3) to 4 x 10^6 FDTPs/Bs was subjected to two
rounds of affinity selection, and then 26 colonies picked
and grown overnight. The phage was then assayed for
lysozyme binding by ELISA (as example 6). Five colonies
yielded phage with lysozyme binding activities, see table 1,
and these were shown to encode the scFv (Dl.3) by PCR
screening (example 13, using 30 cycles of 1 minute at 92°C,
1 minute at 60°C, 1 minute at 72°C using CDR3PCR1 and oligo
3 (fig. 4) as primers).

Thus very rare pAbs can be fished out of large populations, by using antigen to select and then screen the phage.

Methods

Affinity Chromatography of pAbs

Approximately 10^{12} phage particles in 1ml MPBS were loaded onto a 1 ml lysozyme-Sepharose affinity column which had been prewashed in MPBS. The column was washed in turn with 10 ml PBS; then 10 ml 50 mM Tris-HCI, 500 mM NaCl pH 7.5; then 10ml 50 mM Tris-HCI, 500 mM NaCl pH 8.5; then 5 mls 50 mM Tris-HCI, 500 mM NaCl pH 9.5 (adjusted with triethylamine) and then eluted with 5 ml 100 mM triethylamine. The eluate was neutralised with 0.5 M sodium phosphate buffer pH 6.8 and the phage plated for analysis. For a second round of affinity chromatography, the first column eluate was plated to about 30,000 colonies per petri dish. After overnight growth, colonies were then scrape into 5 ml 2 x YT medium, and a 20 μl aliquot diluted into 10 ml fresh medium and grown overnight. The phage was PEG precipitated as above, resuspended in 1 ml MPBS and loaded onto the column, washed and eluted as above.

Oligonucleotides synthesised:

CDR3PCR1 5'TGA GGA C(A or T) C(A or T)GC CGT CTA CTA CTG TGC 3'

Oligonucleotide probing

40 pmole oligonucleotide VH1FOR (Ward, E. S., et al (1989) Nature 341, 544-546), specific to pAb (D1.3) was phosphorylated with 100 μCi y-32P ATP, hybridised (1pmole/ml) to nitrocellulose filters at 67°C in 6 x SSC buffer for 30 minutes and allowed to cool to room temperature for 30 mins, washed 3 x 1 min at 60°C in 0.1 x SSC.

Example 9

Construction of pAb Expressing Anti-hapten Activity

Oxazalone is a hapten that is a commonly used for studying

the detail of the immune response. The anti-oxazalone antibody, NQ11 has been described previously (E. Gherardi, R. Pannell, C. Milstein, J. Immunol. Method 126 61-68). A plasmid containing the VH and VL genes of NQ11 was converted to a ScFv form by inserting the BstEII/SacI fragment of SCFVD1.3 myc (nucleotides 432-499 of Fig 5) between the VH and VL genes to generate pSCFVNQ11, the sequence of which is shown in fig 13. This ScFv was cloned into the Pst1/XhoI site of FdTPs/Xh (as described earlier) to generate pAb NQ11. (NQ11 has an internal Pst1 site and so it was necessary to do a complete digest of pSCFVNQ11 with XhoI followed by a partial digest with Pst1)

The specific binding of pAb NQ11 was confirmed using ELISA. ELISA plates were coated at 37°C in 50mM NaHCO3 at a protein concentration of 200 µg/ml. Plates were coated with either hen egg lysozyme (HEL), bovine serum albumin (BSA), or BSA conjugated to oxazalone (OX-BSA) (method of conjugation in Makela O., Kaartinen M., Pelkonen J.L.T., Karjalainen K. (1978) J.Exp.Med.148 1644). Preparation of phage, binding to ELISA plates, washing and detection was as described in example 6. Samples were assayed in duplicate and the average absorbance after 10 minutes presented in figure 14.

This result demonstrates that the pAb NQ11 binds the correct antigen. Figure 14 also shows that pAb D1.3 and pAb NQ11 bind only to the antigen against which the original antibodies were raised.

Example 10

Enrichment of pAb D1.3 from Mixtures of Other pAb by Affinity Purification

3×10^{10} phage in 10mls of PBSM at the ratios of pAb D1.3 to pAb NQ11 shown in table 2 were passed over a 1ml lysozyme Sepharose column. Washing, elution and other methods were as described in example 8 unless otherwise stated. Elutes from the columns were used to infect TG1 cells which were

then plated out. Colonies were probed with a probe which distinguishes pAb D1.3 from pAb NQ11. The sequence of this oligonucleotide (D1.3CDR3A) is:-

5'GTA GTC AAG CCT ATA ATC TCT CTC 3'

Table 2 presents the data from this experiment. An enrichment of almost 1000 fold was achieved in one round and an enrichment of over a million fold in two rounds of purification. This parallels the result described in example 8.

Example 11

Insertion of the Extracellular Domain of the Human Receptor for Platelet Derived Growth Factor Isoform BB into fdCAT-2

A gene fragment encoding the extracellular domain of the human receptor for platelet derived growth factor isoform BB (h-PDGFB-R) was isolated by amplification using the polymerase chain reaction, of plasmid RP41 (from the American Type Culture collection, Cat. No. 50735), a cDNA clone encoding amino acids 43 to 925 of the PDGF-B receptʟ (Gronwald, R.G.K. et al., PNAS 85, p3435-3439 (1988), amino acids 1 to 32 constitute the signal peptide). The oligonucleotide primers were designed to amplify the region of the h-PDGFB-R gene corresponding to amino acids 43 to 531 of the encoded protein. The primers also incorporate a unique ApaLl site at the 5' end of the fragment and a unique XhoI site at the 3' end, to facilitate cloning into thʟ vector fdCAT-2. The sequence of the primers is:

RPDGF1 5'C ACA GTG CAC GTC CTC AAT GTC TCC AGC ACC TTC 3'
RPDGF2 5'GAT CTC GAG CTT AAA GGG CAA GGA GTG TGG CAC 3'

PCR amplification was performed using high fidelity conditions (Eckert, K.A. and Kunkel, T.A., 1990 Nucl. Acids. Research 18 p3739-3744). The PCR mixture contained; 20mM Tris HCl (pH7.3 at 70°C), 50mM KCl, 4mM magnesium chloride, 0.01% gelatin, 1mM each of dATP, dGTP, dCTP, and dTTP, 500ng/ml RP41 DNA, 1μM each primer and 50 units/ml Taq polymerase (Cetus/Perkin-Elmer, Beaconsfield, Bucks, UK)

Thirty cycles of PCR were performed with denaturation at 92°C for 1 minute, annealing at 50°C for 2 min and extension at 72°C for 3 min. This reaction resulted in amplification of a fragment of ca 1500bp as expected.

fdCAT-2 vector DNA was digested with ApaL1 and Xhol (New England Biolabs) according to manufacturers recommendations, extracted with phenol/chloroform and ethanol precipitated (Sambrook et al., supra). Cloning of amplified RP41 DNA into this vector and identification of the desired clones was performed essentially as in example 12 except that probing was with 32-P labelled RPDGF1 and analytical PCR was performed using RPDGF1 and RPDGF2 as primers.

Example 12

Insertion of the Extracellular Domain of the Human Epidermal Growth Factor Receptor into fd-CAT2

A gene fragment encoding the extracellular domain of the human epidermal growth factor receptor (hEGF-R) was isolated by polymerase chain amplification of plasmid pJ3EGF-R (Clark et al. (1988) J. Cell Physiol. 134, p421-428) which contains the gene for hEGF-R (Ullrich, A. et al., Nature 309, p418-425, (1984). The oligonucleotide primers used were designated to amplify the region of the hEGF-R gene corresponding to amino acid 1 of the mature protein through to amino acid 621. The primers also incorporate an unique ApaL1 site at the 5' end of the fragment and an unique Xho 1 site at the 3' end, to facilitate cloning into the vector fd-CAT2. The sequence of the primers is:

Oligo KSJ4:5' GAT CTC GAG GGA CGG GAT CTT AAG CCC ATT CGT TGG 3'

Oligo KSJ5:5' CAG AGT GCA CTG GAG GAA AAG AAA GTT TGC CA 3'

PCR amplification was carried out using high-fidelity conditions (Eckert, K.A. and Kunkel, T.A. 1990 Nucl. Acids Res. 18, 3739-3744). The PCR mixture contained: 20mM TrisHCl (pH 7.3 at 70°C), 50mM KCl, 4mM $MgCl_2$, 0.01%

gelatin, 1mM each of dATP, dGTP, dCTP and dTTP, 500ng/ml pJ3 plasmid, 0.5μM each primer and 50U/ml Taq polymerase (Cetus/Perkin-Elmer). Thirty cycles of PCR amplification were performed with denaturation at 92°C for 1 minute, annealing at 50°C for 2 minutes and extension at 72°C for 3 minutes. This regime resulted in amplification of a fragment of the expected size (approximately 1800bp).

The PCR mixture was extracted with phenol/chloroform and ethanol precipitated (Sambrook et al. supra.) before digestion with ApaL 1 and Xho 1 (New England BioLabs) according to manufacturers recommendations. The fragment was resolved on a 1% Tris-Acetate-EDTA agarose gel (Sambrook et al. supra.) and purified using Geneclean (BIO 101) (Geneclean, La Jolla, San Diego, California, USA) according to manufacturers reccomendations.

fd-CAT2 vector DNA was digested with ApaL 1 and Xho 1 (New England BioLabs) according to manufacturers reccomendations, extracted with phenol/chloroform and ethanol precipitated (Sambrook et al. supra.).

75ng of ApaL 1/Xho 1-digested vector DNA was ligated to 40ng of PCR-amplified ApaL 1/Xho I-digested hEGF-R fragment in 12μl of ligation buffer (66mM TrisHCl (pH7.6), 5mM MgCl$_2$, 5mM dithiothreitol, 100mg/ml bovine scrum albumin, 0.5mM ATP, 0.5mM Spermidine) and 400 units T4 DNA ligase (New England BioLabs) for 16 hours at 16°C.

Two μl of the ligation mixture was transformed into 200μl of competent E.coli MC1061 cells, plated on 2YT agar containing 15mg/ml tetracycline and incubated at 30°C for 20 hours.

Colonies containing hEGF-R were identified by probing with 32p-labelled KSJ 4 oligonucleotide (Sambrook et al. supra.) and the presence of an insert in hybridising colonies confirmed by PCR using the conditions described above. In this case the template DNA was prepared from the colonies by picking some colony material into 100μl of

distilled water and boiling for 10 minutes. 1μl of this mixture was used in a 20μl PCR.

Example 13.

Insertion of a Gene Encoding an Enzyme (Alkaline phosphatase) into fd-CAT2

As an example of the expression of a functional enzyme on the bacteriophage surface, the applicants have chosen bacterial alkaline phosphatase an enzyme that normally functions as a dimer (McCracken, S. and Meighen, E., J. Biol. Chem. 255, p2396-2404, (1980)). The oligonucleotides were designed to generate a PCR product with an Apa L1 site at the 5' end of phoA gene and a Not 1 site at its 3' end, thus facilitating cloning into fd-CAT 2 to create a gene III fusion protein. The oligonucleotides synthesised were:

phoA1:5' TAT TCT CAC AGT GCA CAA ACT GTT GAA CGG ACA CCA GAA ATG CCT GTT CTG 3' and,

phoA2:5' ACA TGT ACA TGC GGC CGC TTT CAG CCC CAG AGC GGC TTT C3'

The sequence of the phoA gene is presented in Chang C. N. et al., Gene 44, p121-125 (1986).

The PCR reaction was carried out in 100μl containing 50mM KC1, 2.5mM MgCl$_2$, 0.01% gelatin, 10mM Tris/HCl pH 8.3, 0.25 units/μl of Taq polymerase (Cetus/Perkin Elmer) and 0.5μg/ml template. The template was the pEK plasmid (described by Chaidaroglou et al., Biochemistry 27 p8338-8343, 1988). The PCR was carried out in a Techne (Techne, Duxford, Cambridge, UK) PHC-2 dri-block using thirty cycles of 1 min at 92°C, 2 min at 50°C, 3 min at 72°C.

The resultant product was extracted with phenol:chloroform, precipitated with ethanol, and the pellet dissolved in 35μl water. Digestion with 0.3 units/μl of Apl L1 was carried out in 150μl volume according to manufacturers instructions for two hours at 37°C. After heat inactivation of the enzyme at 65°C, NaCl was added to a final concentration of 150mM and 0.4 units/μl Notl enzyme

added. After incubation for 2 hours at 37°C, the digest was extracted with phenol:chloroform and precipitated as above, before being dissolved in 30μl of water. The vector fd-CAT2 was sequentially digested with Apa L1 and Not1 according to the manufacturers instructions and treated with calf intestinal alkaline phosphatase as described in example 2. The sample was extracted three times with phenol:chloroform, precipitated with ethanol and dissolved in water. The ligations were performed with a final DNA concentration of 1-2ng/μl of both the cut fd-CAT2 and then digested PCR product. The ligations were transformed into competent TG1 cells and plated on 2xTY tet plates. Identification of clones containing the desired insert was by analytical PCR performed using the conditions and primers above on boiled samples of the resulting colonies. The correct clone containing the phoA gene fused in frame to gene III was called fd-phoAl. The sequence at the junction of the cloning region is given in figure 15.

<u>Example 14</u>

<u>Measuring Enzyme Activity of Phage-enzyme</u>

Overnight cultures of TG1 or KS272 (E.coli cells lacking phoA. Strauch K. L., and Beckwith J. PNAS 85 1576-1580, 1988) cells containing either fd-phoAl or fd-CAT2 were grown at 37°C in 2xTY with 15μg/ml tetracycline. Concentrated PEG precipitated phage were prepared as described earlier. Enzyme assays (Malamy, M.H. and Horecker B.L., Biochemistry 3, p1893-1897, (1964)) were carried out at 24°C in a final concentration of 1M Tris/HCl pH 8.0, 1mM nitrophenyl phosphate (Sigma), 1mM MgCl2. 100μl of a two times concentrate of this reaction mixture was mixed with 100μl of the test sample in a 96 well plate. Absorbance readings were taken every minute for 30 minutes at a wavelength of 405nm in a Titretek Mk 2 plate reader. Initial reaction rates were calculated from the rate of change of absorbance using a molar absorbance of 17000.

Standard curves (amount of enzyme vs. rate of change of absorbance) were prepared using dilutions of purified bacterial alkaline phosphatase (Sigma type III) in 10mM Tris/HCl pH 8.0, 1mM EDTA. The number of enzyme molecules in the phage samples were estimated from the actual rates of change of absorbance of the phage samples and comparison to this standard curve.

The results in Table 3 show that alkaline phosphatase activity was detected in PEG precipitated material in the sample containing fd-phoA1 but not fd-CAT2. Furthermore, the level of activity was consistent with the expected number of 1-2 dimer molecules of enzyme per phage. The level of enzyme activity detected was not dependant on the host used for growth. In particular, fd-phoA1 grown on phoA minus hosts showed alkaline phosphatase activity.

Therefore, the phage express active alkaline phosphatase enzyme from the phoA-gene III fusion on the phage surface.

Example 15

Insertion of Binding Molecules into Alternative Sites in the Phage

The availability of an alternative site in the phage for the insertion of binding molecules would open up the possibility of expressing more than one antibody fragment in a single pAb. This may be used to generate single or multiple binding specificities. The presence of two distinct binding activities on a single molecule will greatly increase the utility and specificity of this molecule. It may be useful in the binding of viruses with a high mutational rate such as human immunodeficiency virus. In addition, it may be used to bring antigens into close proximity (eg. drug targetting or cell fusion) or it may act as a "molecular clamp" in chemical, immunological or enzymatic processes.

The vector fd-tet and the derivatives described here, have a single BamH1 site in gene 3. This has previously

been used for the expression of peptide fragments on the surface of filamentous bacteriophage (Smith GP. (1985) Science 228 p1315-1317 and de la Cruz et al. (1988) J Biol. Chem. 263 p4318-4322). This provides a potential alternative site for the insertion of antibody fragments.

DNA fragments encoding SCFv's from D1.3 or NQ11 were generated by PCR using the primers shown below. These primers were designed to generate a fragment with BamH1 sites near both the terminii, to enable cloning into the BamH1 site of gene3 (see figure 16(1)). The oligonucleotides used, also ensure that the resulting PCR product lacks Pst1 and Xho1 restriction sites normally used for manipulating the SCFv's (see figure 16(1)). This will facilitate subsequent manipulation of a second antibody fragment in the usual way at the N terminus of gene 3. The oligonucleotides used were:-

G3Bam1 5'TTT AAT GAG GAT CCA CAG GTG CAG CTG CAA GAG 3'
G3Bam2 5' AAC GAA TGG ATC CCG TTT GAT CTC AAG CTT 3'.

Preparation of vector and PCR insert

The PCR reaction was carried out in an 80 µl reaction as described in example 13 using 1ng/µl of template and 0.25U/µl of Taq polymerase and a cycle regime of 94°C for 1 minute, 60°C for 1 minute and 70°C for 2 minutes over 30 cycles. The template was either pSCFvNQ11 (example 9) o- SCFvD1.3 myc (example 2). Reaction products were extracted with phenol:chloroform, precipitated, dissolved in water and digested with BamH1 according to manufacturers instructions. The digest was re-extracted with phenol:chloroform, precipitated and dissolved in water.

The vector FDTPs/Xh was cleaved with BamH1 and treated with calf intestinal phosphatase and purified as described in example 2. Ligations were set up at a vector concentration of approximately 6ng/µl and a PCR insert concentration of approximately 3ng/µl. These were ligated for 2.5 hours at room temperature before transforming into

competent TG1 cells and plating on TYE tet plates. The resultant colonies were probed as described in example 8. DNA was prepared from a number of colonies and the correct orientation and insert size confirmed by restriction digestion with Hind III in isolation or in combination with BamH1. (One Hind III site is contributed by one of the primers and the other by the vector).

Two clones containing a D1.3 insert (FDTBam1 and FDTBam2) and one containing an NQ11 insert (NQ11Bam1) were grown up and phage prepared as described earlier. ELISAs were carried out as described in example 6. No specific signal was found for any of these clones suggesting that the natural BamH1 site is not a suitable site for insertion of a functional antibody (results not shown).

It may be possible to clone into alternative sites to retain binding activity. The peptide repeats present in gene3 may provide such a site (figure 16 blocks A and B). This can be done by inserting a BamH1 site and using the PCR product described above. To facilitate this, the natural BamH1 site was removed by mutagenesis with the oligonucleotide shown below (using an in vitro mutagenesis kit (Amersham International)):-

G3mut6Bam 5' CA AAC GAA TGG GTC CTC CTC ATT A 3'

The underlined residue replaces an A residue thereby removing the BamH1 site. DNA was prepared from a number of clones and several mutants lacking BamH1 sites identified by restriction digestion.

The oligonucleotide G3 Bamlink was designed to introduce a BamH1 site at a number of possible sites within the peptide linker sites A and B, see figure 16(2). The sequence of the linker is:

5'CC (G or A) CC ACC CTC GGA TCC (G or A) CC ACC CTC 3'

Its relationship to the peptide repeats in gene III is shown in figure 16.

Example 16

PCR Assembly of Mouse VH and VL kappa (VLK) Repertoires

for Phage Display

5

The principle is illustrated in figure 17. Details

are provided in sections A to F below but the broad

outline is first discussed.

1.   cDNA is prepared from spleen RNA from an appropriate

10   mouse and the VH and VLK repertoires individually

amplified.   Separately,   primers ~~reverse and~~

~~complementary to VH1FOR 2 (domain 1) and VLK2BACK~~

~~(domain 2)~~ are used to amplify an existing scFv-

containing DNA by PCR.   (The term FOR refers to eg.

15   a primer for amplification of sequences on the sense

strand resulting in antisense coding sequences.   The

term BACK refers to eg. a primer for amplification

of sequences on the antisense strand resulting in

sense coding sequences).   This generates a 'linker'

20   molecule encoding the linker with the amino acid

sequence (1 letter code) $(GGGGS)_3$ which overlaps the

two primary (VH and VLK) PCR products.

2.   The separate amplified VH, VLK and linker sequences

now have to be assembled into a continuous DNA

25   molecule by use of an 'assembly' PCR.   In the

secondary 'assembly' PCR, the VH, VLK and linker

bands are combined and assembled by virtue of the above referred to overlaps, to generate an assembled PCR band with VHs and VLKs randomly spliced in frame for expression as scFVs.

5 The assembly PCR is carried out in two stages. Firstly, 7 rounds of cycling with just the three bands present in the PCR, followed by a further 20 rounds in the presence of the flanking primers VH1BACK ~~(referring to domain 1 of VH)~~ and VLKFOR.

10 The nucleotide sequences for these oligonucleotide primers are provided under the section entitled 'Primer Sequences' below. This two stage process, avoids the potential problem of preferential amplification of the first combinations to be

15 assembled.

3. For cloning into the phage system, the assembled repertoires must be 'tagged' with the appropriate restriction sites. In the example provided below this is illustrated by providing an ApaL1

20 restriction site at the VH end of the continuous DNA molecule and a Not 1 site at the VLK end of the molecule. This is carried out by a third stage PCR using tagged primers. The nucleotide sequences for these oligonucleotide primers are also provided

25 under the section entitled 'Primer Sequences' below. There are however, 4 possible kappa light chain

$\mathcal{J}$ sequences. ~~(whereas there is only a single heavy chain sequence).~~ Therefore 4 oligonucleotide primer sequences are provided for VLK.

5 For this third stage PCR, sets of primers which have 4 and 10 nucleotides after the restriction sites have been used. However, long tags may give better cutting, in which case 15-20 nucleotide overhangs could be used.

Scrupulously clean procedures must be used at all

10 times to avoid contamination during PCR. Negative controls containing no DNA must always be included to monitor for contamination. Gel boxes must be depurinated. A dedicated Geneclean kit (B10 101, Geneclean, La Jolla, San Diego, California, USA) can be

15 used according to manufacturers instructions to extract DNA from an agarose gel. The beads, NaI and the NEW wash should be aliquoted.

All enzymes were obtained from CP Laboratories, P.O. Box 22, Bishop's Stortford, Herts CM20 3DH and the

20 manufacturers recommended and supplied buffers were used unless otherwise stated.

A. RNA Preparation

RNA can be prepared using many procedures well known

25 to those skilled in the art. As an example, the following protocol (Triton X-100 lysis, phenol/SDS RNase

ribonucleoside vanadyl

inactivation) gives excellent results with spleen and hybridoma cells (the addition of ~~NRG~~ RVC (~~veronal ribocyl~~ complex) as an RNase inhibitor is necessary for spleen cells). Guanidinium isothiocyanate/CsCl procedures

5   (yielding total cellular RNA)also give good results but are more time-consuming.

1.   Wash 1 - 5 x 10^7 freshly harvested cells in 50 ml PBS at 800g for 10 minutes.

2.   On ice, add 1 ml ice-cold lysis buffer (see later

10   for details) to the pellet and resuspend it with a 1 ml Gilson pipette. Leave on ice for 5 minutes.

3.   Spin for 5 minutes at 4°C in a microfuge at 13000 rpm, in precooled tubes.

4.   Transfer 0.5 ml of the supernatant to each of two

15   eppendorf tubes containing 60µl 10% (w/v) SDS and 250µl phenol (equilibrated with 100mM Tris-HCl pH 8.0). Vortex hard for 2 minutes, then microfuge (13000rpm) for five minutes at room temperature.

5.   Re-extract the aqueous upper phage five times with

20   0.5 ml of phenol.

6.   Precipitate with 1/10 volume 3M sodium acetate and 2.5 volumes ethanol at 20°C overnight or dry ice-isopropanol for 30 minutes.

7.   Wash the RNA pellet and resuspend in 50µl water.

25   Use 2.5 µl to check concentration by measuring the optical density at 260nm and check 2µg on a 1% agarose gel. 40µg

, New England Biologicals Ltd. as supplied by C. P. Laboratories Ltd., address given above, catalogue no. 1402

to 107μg of RNA was obtained from spleen cells derived from mice.

Lysis buffer is [10mM Tris-HCl pH 7.4, 1mM MgCl$_2$, 150mM NaCl, 10mM RVC (New England Biolabs), 0.5% (w/v) Triton X-100], prepared fresh.

B.   cDNA Preparation

cDNA can be prepared using many procedures well know to those skilled in the art. As an example, the following protocol can be used:

1.   Set up the following reverse transcription mix:

	μl
H$_2$O (DEPC-treated)	20
5mM dNTP	10
10 x first strand buffer	10
0.1M DTT	10
FOR primer(s) (10 pmol/μl)	2 (each) (see below)
RNasin (Promega; 40 U/μl)	4

NB

i)   DEPC is diethylpyrocarbonate, the function of which is to inactivate any enzymes that could degrade DNA or RNA

ii)   dNTP is deoxynucleotide triphosphate

iii) DTT is dithiothreitol the function of which is as an

antioxidant to create the anaerobic environment necessary for enzyme function.

iv) RNasin is a ribonuclease inhibitor obtained from Promega Corporation, 2800 Woods Hollow Road, Madison, Wisconsin, USA.

2. Dilute 10 µg RNA to 40 µl final volume with DEPC-treated water. Heat at 65°C for 3 minutes and hold on ice for one minute (to remove secondary structure).

3. Add to the RNA the reverse transcription mix (58 µl) and 4 µl of the cloned reverse transcriptase 'Super RT' (Anglian Biotech Ltd., Whitehall House, Whitehall Road, Colchester, Essex) and incubate at 42°C for one hour.

4. Boil the reaction mix for three minutes, cool on ice for one minute and then spin in a microfuge to pellet debris. Transfer the supernatant to a new tube.

10 x first strand buffer is [1.4M KCl, 0.5M Tris-HCl pH 8.1 at 42°C 80mM MgCl$_2$].

The primers anneal to the 3' end *of the RNA*. Examples of kappa light chain primers are MJK1FONX, MJK2FONX, MJK4FONX and MJK5FONX (provided under 'Primer Sequences' below) and examples of heavy chain primers are CTG GAC AGG GAT CCA

GAG TTC CA and CTG GAC AGG GCT CCA TAG TTC CA. The two heavy chain primers are provided as alternatives, the four light chain primers are provided for kappa light chains 1-4. Primers annealing to CH1, VLK and VL domains could also be used.

C. Primary PCRs

For each PCR and negative control, the following reactions are set up. In the following, the Vent DNA polymerase sold by C.P. Laboratories Ltd. (New England Biolabs) address given above. The buffers are as provided by C.P. Laboratories.

	μl
H_2O	32.5
10 x Vent buffer	5
20 x Vent BSA	2.5
5mM dNTPs	1.5
FOR primer 10 pMol/μl)	2.5
BACK primer 10pmol/μl)	2.5

The FOR and BACK primers are given in the section below entitled 'Primer Sequences'. For VH, the FOR primer is VH1FOR-2 and the BACK primer is VH1BACK. For VLK the FOR primers are MJK1FONX, MJK2FONX, MJK4FONX and MJK5FONX (for the four respective kappa light chains) and the BACK primer is VK2BACK. Only one kappa light chain BACK

primer is necessary, because binding is to a nucleotide sequence common to the four kappa light chains.

UV this mix 5 minutes. Add 2.5 µl cDNA preparation (from B above), 2 drops paraffin oil (Sigma Chemicals,

5 Poole, Dorset, UK). Place on a cycling heating block, e.g. PHC-2 manufactured by Techne Ltd. Duxford UK, pre-set at 94°C. Add 1µl Vent DNA polymerase under the paraffin. Amplify using 25 cycles of 94°C 1 min, 72°C 2 min. Post-treat at 60°C for 5 min.

10 Purify on a 2% lmp (low melting point agarose/TAE (tri-acetate EDTA) gel and extract the DNA to 20 µl H_2O per original PCR using a Geneclean kit (see earlier) in accordance with the manufacturers instructions.

15 D. Preparation of linker

Set up in bulk (eg. 10 times):

	µl
H_2O	34.3
10 x Vent Buffer	5
20 x Vent BSA	2.5
5mM dNTPs	2
LINKFOR primer (10 pmol/µl)	2.5
LINKBACK primer (10 pmol/µl)	2.5
DNA from ~~for D1-3~~ (example 2)	1
Vent enzyme	0.2

20

25

FDTSCVFVD1.3

The FOR and BACK primers are given in the section below entitled 'Primer Sequences'. The FOR primer is LINKFOR and the BACK primer is LINKBACK.

5   Cover with paraffin and place on the cycling heating block (see above) at 94°C. Amplify using 25 cycles of 94°C 1 min, 65°C 1 min, 72°C 2 min. Post-treat at 60°C for 5 min.

Purify on 2% lmp/TAE gel (using a loading dye
10   without bromophenol blue as a 93bp fragment is desired) and elute with SPIN-X column (Costar Limited, 205 Broadway, Cambridge, Ma. USA.,) and precipitation.
Take up in 5µl H_2O per PCR reaction.

15   E.   Assembly PCRs

A quarter of each PCR reaction product (5µl) is used for each assembly. The total volume is 25µl.

For each of the four VLK primers, the following are set up:

20

H_2O	4.95
10 x Vent buffer	2.5
20 X Vent BSA	1.25
5mM dNTPs	0.8

25   UV irradiate this mix for 5 min. Add 5µl each of Vh and Vk band and 1.5µl of linker as isolated from the

preparative gels and extracted using the Geneclean kit as described in C and D above. Cover with paraffin. Place on the cycling heating block preset at 94°C. Add 1μl Vent under the paraffin. Amplify using 7 cycles of 94°C

5   2 min, 72°C 4 min. Then return the temperature to 94°C.

Add 1.5μl each of VH1BACK and the appropriate VKFOR primers MJK1FONX, MJK2FONX, MJK4FONX or MJK5FONX (10 pmol/μl) at 94°C. The primers should have been UV-

10   treated as above. Amplify using 20 cycles of 94°C 1.5 min, 72°C 2.5 min. Post-treat at 60°C for 5 min. Purify on 2% lmp/TAE gel and extract the DNA to 20μl H_2O per assembly PCR using a Geneclean kit (see earlier) in accordance with the manufacturers instructions.

15

F.   Adding Restriction Sites

For each assembly and control set up:

	μl
20 H_2O	36.5
10 x Taq buffer	5
5mM dNTPs	2
FOR primer (10 pmol/μl)	2.5
BACK primer (10 pmol/μl)	2.5
25 Assembly product	1

The FOR and BACK primers are given in the section below entitled 'Primer Sequences'. The FOR primer is any of JK1NOT10, JK2NOT10, JK4NOT10 or JK5NOT10 (for the four respective kappa light chains) for putting a Notl

5   restriction site at the VLK end. The BACK primer is HBKAPA10 for putting an ApaL1 restriction site at the VH end.

Cover with paraffin and place on the cycling heating block preset at 94°C. Add 0.5 μl Cetus Taq DNA

10   polymerase (Cetus/perkin-Elmer, Beaconsfield, Bucks, UK) under the paraffin. Amplification is carried out using 11 to 15 rounds of cycling (depends on efficiency) at 94°C 1 min, 55°C 1 min, 72°C 2 min. Post-treat at 60°C for 5 min.

15   10 x Taq buffer is [0.1M Tris-HCl pH 8.3 at 25°C, 0.5M KCl, 15mM $MgCl_2$, 1mg/ml gelatine].

G.   Work-up

Purify once with $CHCl_3$/IAA (isoamylalcohol), once

20   with phenol, once with $CHCl_3$/IAA and back-extract everything to ensure minimal losses. Precipitate and wash twice in 70% EtOH. Dissolve in 70μl H_2O.

Digest overnight at 37°C with NotI: μl

25   DNA (joined seq)   70

NEB NotI buffer x 10   10

NEB BSA x 10	10
Notl (10 U/µl)	10

The DNA (joined sequence) above refers to the assembled

DNA sequence comprising in the 5' to 3' direction

5   ApaLl restriction site

          VH sequence

          Linker sequence

          VLK sequence

          Not 1 restriction site.

10   The VLK sequence may ~~be~~ contain any one of four possible

kappa J chain sequences.

The enzymes Not 1 above, ApaLl below and the buffers

NEB Not 1, NEB BSA above and the NEB buffer 4 (below) are

obtainable from CP Laboratories, New England Biolabs

15 mentioned above.

Re-precipitate, take up in 80µl H_2O. Add to this

10µl NEB buffer 4 and 10µl Apal 1.

Add the enzyme ApaLl in aliquots throughout the day,

as it has a short half-life at 37°C.

20   Purify on 2% lmp/TAE gel and extract the DNA using a

Geneclean kit, in accordance with the manufacturers

instructions. Redigest if desired.

F.   Final DNA product

25   The final DNA product is an approximate 700 bp

fragment with Apa Ll and Notl compatible ends consisting

of randomly associated heavy and light chain sequences
linked by a linker. A typical molecule of this type is
the scFvD1.3 molecule incorporated into fdscFvD1.3
described in example 3. These molecules can then be

5   ligated into suitable fd derived vectors, e.g. fdCAT2 ~~or~~
~~fd CAT3 (example 1)~~, using standard techniques.

Primer sequences.

W = A or T   R = A or G   S = G or C   M = A or C

10   Primary PCR oligos (restriction sites underlined):

VH1FOR-2   TGA GGA GAC <u>GGT GAC CGT</u> GGT CCC TTG GCC CC

VH1BACK   AGG TSM ARC TGC AGS AGT CWG G

MJK1FONX   CCG TTT GAT TTC CAG CTT GGT GCC

MJK2FONX   CCG TTT TAT TTC CAG CTT GGT CCC

15   MJK4FONX   CCG TTT TAT TTC CAA CTT TGT CCC

MJK5FONX   CCG TTT CAG CTC CAG CTT GGT CCC

VK2BACK   GAC ATT <u>GAG CTC</u> ACC CAG TCT CCA

PCR oligos to make linker:

20   LINKFOR   TGG AGA CTC GGT <u>GAG CTC</u> AAT GTC

LINKBACK   GGG ACC ACG GTC ACC GTC TCC TCA

For adding restriction sites:

HBKAPA10   CAT GAC CAC <u>AGT GCA CAG</u> GTS MAR CTG CAG SAG TCW

25   GG

JK1NOT10   GAG TCA TTC <u>TGC GGC</u> CGC CCG TTT GAT TTC CAG CTT

```
                    GGT  GCC

JK2NOT10    GAG  TCA  TTC  TGC  GGC  CGC  CCG  TTT  TAT  TTC  CAG  CTT

            GGT  CCC

JK4NOT10    GAG  TCA  TTC  TGC  GGC  CGC  CCG  TTT  TAT  TTC  CAA  CTT

            TGT  CCC

JK5NOT10    GAG  TCA  TTC  TGC  GGC  CGC  CCG  TTT  CAG  CTC  CAG  CTT

            GGT  CCC
```

Example 17

Insertion of the Extra/cellular Domain of the Human
Receptor for Platelet Derived Growth FActor PDGF isoform
BB into fdCAT2

This example is substantially equivalent to example
11.   However, in example 11, amino acids 33-42 of the
mature protein were not provided.   In this example, the
primer RPDGF3 also includes bases encoding these amino
acids.

A gene fragment encoding the extracellular domain of
the human receptor for platelet derived growth factor
isoform BB (h-PDGFB-R) was isolated by amplification,
using the polymerase chain reaction, of plasmid RP41
(from the American Type Culture collection, Cat. No.
50735), a cDNA clone encoding amino-acids 43 to 925 of
the PDGF-B receptor (Gronwald, R.G.K. et al PNAS 85
p3435-3439 (1988), amino acids 1 to 32 constitute the
signal peptide).   The oligonucleotide primers were

designed to amplify the region of the h-PDGFB-R gene corresponding to amino acids 43 to 531 of the encoded protein. The primer RPDGF3 for the N-terminal region also included bases encoding amino acids 33 to 42 of the

5 h-PDGFB-R protein (corresponding to the first ten amino acids from the N-terminus of the mature protein) to enable expression of the complete extracellular domain. The primers also incorporate a unique ApaL1 site at the N-terminal end of the fragment and a unique XhoI site at

10 the C terminal end to facilitate cloning into the vector .fdCAT2. The sequence of the primers is:

RPDGF3 5' CAC AGT GCA CTG GTC GTC ACA CCC CCG GGG CCA GAG
        CTT GTC CTC AAT GTC TCC AGC ACC TTC GTT CTG 3'

15

RPDGF2 5' GAT CTC GAG CTT AAA GGG CAA GGA GTG TGG CAC 3'

PCR amplification was performed using high fidelity conditions (Eckert, K.A. and Kunkel, T.A. 1990 Nucl Acids

20 Research 18 3739-3744). The PCR mixture contained: 20mM TrisHCl (pH7.3 at 70°C, 50mM KCl, 4mM magnesium chloride, 0.01% gelatin, 1mM each of dATP, dCTP, dGTP and dTTP, 500ng/ml RP41 DNA, 1μM each primer and 50units/ml Taq polymerase (Cetus/Perkin Elmer, Beaconsfield, Bucks,

25 U.K.). Thirty cycles of PCR were performed with denaturation at 92°C for 1 min, annealing at 60°C for 1

min and extension at 72°C for 1.5 min. This reaction resulted in amplification of a fragment of ca. 1500bp as expected.

fdCAT2 vector DNA (see example 5) was digested with ApaLl and Xhol (New England Biolabs) according to manufacturers recommendations, extracted with phenol/chloroform and ethanol precipitated (Sambrook et al, supra). Cloning of amplified RP41 DNA into this vector and identification of the desired clones was performed essentially as in example 12 except that probing was with 32-P labelled RPDGF2 and analytical PCR was performed using RPDGF3 and RPDGF2.

Example 18

Binding of ^{125}I-PDGF-BB to the Extracellular Domain of the Human Receptor for Platelet Derived Growth Factor Isoform BB Displayed on the Surface of fd Phage. Measured using an Immunoprecipitation Assay.

Phage particles, expressing the extracellular domain of the human platelet derived growth factor isoform BB receptor (fd h-PDGFB-R), were prepared by growing E.coli MC1061 cells transformed with fd h-PDGFB-R in 50ml of 2xTY medium with 15ug/ml tetracyclin for 16 to 20 hours. Phage particles were concentrated using polyethylene glycol as described in example 6 and resuspended in PDGF binding buffer (25mM HEPES, pH7.4, 0.15mM NaCl, 1mM

magnesium chloride, 0.25% BSA) to 1/33rd of the original volume. Residual bacteria and undissolved material were removed by spinning for 2 min in a microcentrifuge. Immunoblots using an antiserum raised against gene III

5   (Prof. I. Rashed, Konstanz., Germany) show the presence in such phage preparations of a geneIII-h-PDGFB-R protein of molecular mass 125000 corresponding to a fusion between h-PDGFB-R external domain (55000 daltons) and geneIII (apparent molecular mass 70000 on SDS-

10   polyacrylamide gel).

     Duplicate samples of 350µl concentrated phage were incubated with ^{125}I-PDGF-BB (78.7fmol, 70nCi, 882Ci/mmol; Amersham International plc, Amersham, Bucks) for 1 hour at 37°C. Controls were included in which fdTPs/Bs vector

15   phage (figure 4(26)) or no phage replaced fd h-PDGFB-R phage. After this incubation, 10ul of sheep anti-M13 polyclonal antiscrum (a gift from M. Hobart) was added and incubation continued for 30 min at 20°C. To each sample, 40ul (20ul packed volume) of protein G Sepharose

20   Fast Flow (Pharmacia, Milton Keynes) equilibrated in PDGF binding buffer was added. Incubation was continued for 30 min at 20°C with mixing by end over end inversion on a rotating mixer. The affinity matrix was spun down in a microcentrifuge for 2 min and the supernatant removed by

25   aspiration. Non-specifically bound ^{125}I-PDGF-BB was removed by resuspension of the pellet in 0.5ml PDGF

binding buffer, mixing by rotation for 5 min, centrifugation and aspiration of the supernatant, followed by two further washes with 0.5ml 0.1% BSA, 0.2% Triton-X-100. The pellet finally obtained was

5   resuspended in 100ul PDGF binding buffer and counted in a Packard gamma counter. For displacement studies, unlabelled PDGF-BB (Amersham International) was added to the stated concentration for the incubation of ^{125}I-PDGF-BB with phage.

10   ^{125}I-PDGF-BB bound to the fd h-PDGFB-R phage and was immunoprecipitated in this assay. Specific binding to receptor phage was 3.5 to 4 times higher than the non-specific binding with vector phage fdTPs/Bs or no phage (fig 19). This binding of ^{125}I-PDGF-BB could be

15   displaced by the inclusion of unlabelled PDGF-BB in the incubation with phage at 37°C (fig 20). At 50nM, unlabelled PDGF-BB the binding of ^{125}I-PDGF-BB was reduced to the same level as the fdTPs/Bs and no phage control. Figure 21 shows the same data, but with the

20   non-specific binding to vector deducted.

These results indicate that a specific saturable site for ^{125}I-PDGF-BB is expressed on fd phage containing cloned h-PDGFB-R DNA. Thus, the phage can display the functional extracellular domain of a cell surface

25   receptor.

Example 19, Construction of Binding molecule-Gene III Phagemid

It would be useful to improve the transfection efficiency of the phage-binding molecule system and also to have the possibility of displaying different numbers and specificities of binding molecules on the surface of the same bacteriophage. The applicants have devised a method that achieves both aims.

The approach is derived from the phagemid system based on pUC119 [Vieira, J and Messing, J. (1987) Methods Enzymol. 153:3]. In brief, gene III from fd-CAT2 (example 5) and fd-CAT2 ScFv D1.3 (example 2) was cloned downstream of the lac promoter in pUC119 in order that the mutated gene III could be 'rescued' by M13K07 helper phage [Vieira, J and Messing, J. et supra.]. The majority of rescued phage would be expected to contain a genome derived from the pUC119 plasmid that contains the binding molecule-gene III fusion and should express varying numbers of the binding molecule on the surface up to the normal maximum of 3-5 molecules of gene III on the surface of wild type phage. The system has been exemplified below using an antibody as the binding molecule.

An fdCAT2 containing the single chain Fv form of the D1.3 antilysozyme antibody was formed by digesting FDTSCFVD1.3 (example 2) with Pstl and Xhol, purifying the

fragment containing the scFv fragment and ligating this into Pstl and Xhol digested fdCAT2. The appropriate clone, called fdCAT2 scFvD1.3 was selected after plating onto 2xTY tetracyclin (15μg/ml) and confirmed by

5   restriction enzyme and sequence analysis.

Gene III from fd-CAT2 (example 5) and fd-CAT2 ScFv D1.3 was PCR-amplified using the primers shown below:

Primer A: TGC GAA GCT TTG GAG CCT TTT TTT TTG GAG ATT TTC AAC G

10   Primer B: CAG TGA ATT CTT ATT AAG ACT CCT TAT TAC GCA GTA TGT TAG C

Primer A anneals to the 5' end of gene III including the ribosome binding site and incorporates a Hind III site. Primer B anneals to the 3' end of gene III at the

15   C-terminus and incorporates two UAA stop codons and an EcoR1 site. One hundred ng of fd-CAT2 and fd-CAT2 ScFv D1.3 DNA was PCR-amplified in a total reaction volume of 50μl as described in example 11, except that 20 cycles of amplification were performed: 94°C 1 minute, 50°C 1

20   minute, 72°C 3 minutes. This resulted in amplification of the expected 1.2Kb fragment from fd-CAT2 and a 1.8Kb fragment from fd-CAT2 ScFv D1.3.

The PCR fragments were digested with EcoR1 and Hind III, gel-purified and ligated into EcoR1- and Hind III-

25   cut and dephosphorylated pUC119 DNA and transformed into E.coli TG1 using standard techniques (Sambrook et al., et

supra).   Transformed cells were plated on SOB agar
containing 100µg/ml ampicillin and 2% glucose.   The
resulting clones were called pCAT-3 (derived from fd-
CAT2) and pCAT-3 ScFv D1.3 (derived from fd-CAT2 ScFv
5   D1.3).

Example 20, Rescue of Anti-Lysozyme Antibody Specificity
from pCAT-3 ScFv D1.3 by M13KO7

    Single CAT-3 and CAT-3 ScFv D1.3 colonies were
10 picked into 1.5ml 2YT containing 100µg/ml ampicillin and
2% glucose, and grown 6hrs at 30°C.   30µl of these
stationary cells were added to 6mls 2YT containing
100µg/ml ampicillin and 2% glucose in 50ml polypropylene
tubes (Falcon, Becton Dickinson Labware, 1950 Williams
15 Drive, Oxnard, CA. USA) and grown for 1.5hrs at 30°C at
380rpm in a New Brunswick Orbital Shaker (New Brunswick
Scientific Ltd., Edison House 163 Dixons Hill Road, North
Mimms. Hatfield, UK).   Cells were pelleted by
centrifugation at 5,000g for 25 minutes and the tubes
20 drained on tissue paper.   The cell pellets were then
resuspended in 6mls 2YTamp containing 100µg/ml ampicillin
(no glucose) and 4mls 2YT containing 1.25×10^9 p.f.u. ml^{-1}
M13KO7 bacteriophage added.   The mixture was left on ice
for 5 minutes followed by growth at 35°C for 45 minutes
25 at 450rpm.   A cocktail was then added containing 4µl
100µg/ml ampicillin, 0.5µl 0.1M IPTG and 50µl 10mg/ml

Kanamycin, and the cultures grown overnight at 35°C, 450rpm.

The following day the cultures were centrifuged and phage particles PEG precipitated as described in example

5   6. Phage pellets were resuspended in 100μl TE (tris-EDTA see example 6) and phage titred on E.coli TG1. Aliquots of infected cells were plated on 2YT containing either 100μg/ml ampicillin to select for pUC119 phage particles, or 50μg/ml Kanamycin to select for the M13 KO7

10 helper phage. Plates were incubated overnight at 37°C and antibiotic-resistant colonies counted:

DNA	amp^R	Kan^R
pCAT-3	1.8×10^{11} colonies	1.2×10^9 colonies
pCAT-3ScFv D1.3	2.4×10^{11} colonies	2.0×10^9 colonies

15 This shows that the amp^R phagemid particles are infective and present in the rescued phage population at a 100-fold excess over Kan^R M13KO7 helper phage.

Phage were assayed for anti-lysozyme activity by ELISA as described in example 6, with the following

20 modifications:

1)   ELISA plates were blocked for 3 hrs with 2% Marvel/PBS

2)   50μl phage, 400μl 1xPBS and 50μl 20% marvel were mixed end over end for 20 minutes at room temperature

25 before adding 150μl per well.

3)   Phage were left to bind for 2 hours at room

temperature.

4)   All washes post phage binding were:

2 quick rinses PBS/0.5% TWEEN 20

3x2 minute washes   -----"-----

5   2 quick rinses PBS no detergent

3x2 minute washes   -----"-----

The result of this ELISA is shown in figure 22, which shows that the anibody specificity can indeed be rescued efficiently.

10   It is considered a truism of bacterial genetics that when mutant and wild-type proteins are co-expressed in the same cell, the wild-type protein is used preferentially. This is analagous to the above situation wherein mutant (i.e. antibody fusion) and wild-type gene III proteins (from M13KO7) are competing for assembly as part of the pUC119 phagemid particle. It is therefore envisaged that the majority of the resulting pUC 119 phage particles will have fewer gene III-antibody fusion molecules on their surface than is the case for the purely phage system described for instance in example 2. Such phagemid antibodies are therefore likely to bind antigen with a lower avidity than fd phage antibodies with three or more copies of the antibody fusion on their surfaces (there is no wild-type gene III protein in the system described, for instance, in example 2), and provide a route to production of phage particles with

different numbers of the same binding molecule (and hence different avidities for the ligand/antigen) or multiple different binding specificities on their surface, by using helper phage such as M13KO7 to rescue cells

5) expressing two or more gene III-antibody fusions.

It is also possible to derive helper phage that do not encode a functional gene III in their genomes (by for example deleting the gene III sequence or a portion of it or by incorporating an amber mutation within the gene).

10 These defective phages will only grow on appropriate cells (for example that provide functional gene III in trans, or contain an amber suppressor gene), but when used to rescue phage antibodies, will only incorporate the gene III antibody fusion encoded by the phagemid into

15 the released phage particle.

Example 21.   Transformation Efficiency of pCAT-3 and pCAT-3 ScFv D1.3 phagemids

PUC 19, pCAT-3 and pCAT-3 ScFv D1.3 plasmid DNAs,

20 and fdCAT-2 phage DNA was prepared, and used to transform E.coli TG1, pCAT-3 and pCAT-3 ScFv D1.3 transformations were plated on SOB agar containing 100µg/ml ampicillin and 2% glucose, and incubated overnight at 30°C. fdCAT-2 transformations were plated on 2YT agar containing

25 15µg/ml tetracycline and incubated overnight at 37°C. Transformation efficiences are expressed as colonies per

µg of input DNA.

DNA	Transformation efficiency
pUC 19	1.10^9
pCAT-3	1.10^8
pCAT-3ScFv D1.3	1.10^8
fd CAT-2	8.10^5

As expected, transformation of the phagemid vector is approximately 100-fold more efficient than the parental fdCAT-2 vector. Furthermore, the presence of a ScFv antobdy fragment does not compromise efficiency. This improvement in transformation efficiency is practically useful in the generation of phage antibody libraries that have large repertoires of different binding specificities.

Table 1. Enrichment of pAb (D1.3) from vector population

INPUT RATIO[a]	OUTPUT RATIO		ENRICHMENT[d]
	oligo[b]	ELISA[c]	
pAb : fd-CAT1	pAb : total phage	pAb : total phage	
Single Round			
1 : 4x10³	43/124		1.3 x 10³
1 : 4x10⁴	2/82		1.0 x 10³
Two Rounds			
1 : 4x10⁴	197/372		2.1 x 10⁴
1 : 4x10⁵	90/356	3/24	1.0 x 10⁵
1 : 4x10⁶	27/183	5/26	5.9 x 10⁵
1 : 4x10⁷	13/278		1.8 x 10⁶

Footnotes: [a]Approximately 10^{12} phage with the stated ratio of pAb (D1.3) : FDTPs/Bs were applied to 1 ml lysozyme-sepharose columns, washed and eluted. [b]TG1 cells were infected with the eluted specific binding phage and plated onto TY-tet plates. After incubation overnight at 30-37°C the plates were analysed by hybridisation to the ^{32}P-labelled oligonucleotide VH1FOR (Ward et al op cit) which is specific to pAb D1.3. [c]Single colonies from overnight plates were grown, phage purified, and tested for lysozyme binding. [d]Enrichment was calculated from the oligonucleotide probing data.

Table 2 Enrichment of pAb(D1.3) from mixed pAb population

Input Ratio[1] (pAbD1.3:pAbNQ11)	Output Ratio[2] (pAb D1.3: Total phage)	Enrichment
Single Round		
$1 : 2.5 \times 10^4$	18/460	0.98×10^3
$1 : 2.5 \times 10^5$	3/770	0.97×10^3
$1 : 2.5 \times 10^6$	0/112	
pAb NQ11 only	0/460	
Second Round		
$1 : 2.5 \times 10^4$	119/170	1.75×10^4
$1 : 2.5 \times 10^5$	101/130	1.95×10^5
$1 : 2.5 \times 10^6$	102/204	1.26×10^6
$1 : 2.5 \times 10^7$	0/274	
$1 : 2.5 \times 10^8$	0/209	
pAb NQ11 only	0/170	

Notes

1. 10^{10} phage applied to a lysozyme column as in table 1.

2. Plating of cells and probing with oligonucleotide as in table 1, except the oligonucleotide was D1.3CDR3A

Table 3: Enzymic activity of phage-enzyme

Input	ng of enzyme or No. of phage	Rate (OD/hr)	No. of molecules of Enzyme equivalent $(x10^{-11})$
Pure Enzyme	335	34	24.5
Pure Enzyme	177.5	17.4	12.25
Pure Enzyme	88.7	8.7	6.125
Pure Enzyme	44.4	4.12	3.06
Pure Enzyme	22.2	1.8	1.5
Pure Enzyme	11.1	0.86	0.76
No Enzyme	0	0.005	0
fd-phoA1/TG1	1.83×10^{11}	5.82	4.2
fd-CAT2/TG1	1.0×10^{12}	0.155	0.112
fd-phoA1/KS272	7.1×10^{10}	10.32	7.35
fd-CAT2/KS272	8.2×10^{12}	0.038	0.027

FIGURE 1: Antibody structure

Antigen binding site

Fab

Fv

The single domain antibody (dAb, Ward et al. 1989) consists of a single V_H domain.

FIGURE 2: ASSAY FORMATS

2 i) Binding/elution

2 ii) Competion

p - Phage antibody population to be sampled.
ag - Antigen to which binding required.
c - Competitor antibody/ phAb/ligand etc population.
s - Surface (eg plastic, beads etc).
d - Detection system

III (adsorption protein)

ClaI

VIII (major coat protein)

scale: 1 inch = 1000 bases

FDTET

ClaI

BstEII

BstEII

ClaI

tetracycline resistance protein (tetA)

fd-tet

⌄

cleave with BstEII

⌄

fill in with Klenow

⌄

re-ligate

▼

FDTδBst

⌄

in vitro mutagenesis (oligo1)

▼

FDTPs/Bs

⌄

in vitro mutagenesis (oligo 2)

▼

FDTPs/Xh

Figure 3 Scheme for construction of vectors

1

(1653)
Oligo 1   ACA ACT TTC AAC AGT TGA GGA GAC GGT GAC CGT AAG CTT CTG CAG TTG GAC CTG AGC
GGA GTG AGA ATA (1620)

(1653)
Oligo 2   ACA ACT TTC AAC AGT TTC CCG TTT GAT CTC GAG CTC CTG CAG TTG GAC CTG

(1704)
Oligo 3   GTC GTC TTT CCA GAC GTT AGT

2

GENE III   GENE III

SIGNAL
CLEAVAGE SITE

(1624)   (1650)
A TCT CAC TCC GCT _____   GAA ACT GTT GAA AGT

        Q   V   Q   L   Q   V   T   V   S   S
B TCT CAC TCC GCT CAG GTC CAA CTG CAG AAG CTT ACG GTC ACC GTC TCC TCA ACT GTT GAA AGT
                            PstI   BstEII

        Q   V   Q   L   Q   L   E   I   K   R
C TCT CAC TCC GCT CAG GTC CAA CTG CAG GAG CTC GAG ATC AAA CGG   GAA ACT GTT GAA AGT
                            PstI   XhoI

β = FDTPs/Bs
C = FDTPs/Xh

Figure 4. Sequence of oligos and vectors

Figure 5. Sequence of SCFvD1.3myc

rbs

GCATTCAAATTCTATTTCAAGGAGACAGTCATAATGAAATACCTATTGCCTACGGCAGCC

10   20   30   40   50   60

SphI

PelB leader

GCTGGATTGTTATTACTCGCTGCCCAACCAGCGATGGCCCAGGTGCAGCTTCAGG....TCA

70   80   90   100   110   120

PstI

G P G L V A P S Q S L S I T C T V S G F

GGACCTGGCCTGGTGGCGCCCTCACAGAGCCTGTCCATCACATGCACCGTCTCAGGGTTC

130   140   150   160   170   180

S L T G Y G V N W V R Q P P G K G L E W

TCATTAACCGGCTATGGTGTAAACTGGGTTCGCCAGCCTCCAGGAAAGGGTCTGGAGTGG

190   200   210   220   230   240

VHD1.3

CTGGGAATGATTTGGGGTGATGGAAACACAGACTATAATTCAGCTCTCAAATCCAGACTG

250   260   270   280   290   300

AGCATCAGCAAGGACAACTCCAAGAGCCAGTTTTTCTTAAAAATGAACAGTCTGCACACT

310   320   330   340   350   360

GATGACACAGCCAGGTACTACTGTGCCAGAGAGAGAGATTATAGGCTTGACTACTGGGGC

370   380   390   400   410   420

Linker Peptide

CAAGGCACCACGGTCACCGTCTCCTCAGgtggaggcggttcaggcggaggtggctctggc

430   440   450   460   470   480

BstEII

ggtggcggatcgGACATCCAGTTGACTCAGTCTCCAGCCTCCTTCTGCGTCTGTGGGA

490   500   510   520   530   540

SacI

GAAACTGTCACCATCACATGTCGAGCAAGTGGGAATATTCACAATTATTTAGCATGGTAT

550   560   570   580   590   600

CAGCAGAAACAGGGAAAATCTCCTCAGCTCCTGGTCTATTATACAACAACCTTAGCAGAT

610   620   630   640   650   660

VKD1.3

GGTGTGCCATCAAGGTTCAGTGGCAGTGGATCAGGAACACAATATTCTCTCAGATCAAC

670   680   690   700   710   720

AGCCTGCAACCTGAAGATTTTGGGAGTTATTACTGTCAACATTTTTGGAGTACTCCTCGG

730   740   750   760   770   780

Myc Tag (TAG1)

ACGTTCGGTGGAGGCACCAAGCTCGAGATCAAACGGGAACAAAAACTCATCTCAGAAGAG

790   800   810   820   830   840

XhoI

GATCTGAATTAATAATGATCAAACGGTAATAAGGATCCAGTTCGCATTC

850   860   870   880

EcoRI

Fig. 6.

Binding of phage antibodies to lysozyme:
varying amount of supernatant

□ ⋯⋯ FDISCFVD1.3
○ — FDIVHD1.3
△ ⋯ FDIPstXh

O.D.
405nm

Volume of phage supernatant (mls)

Methods as described in example 4

Fig. 7

Binding of phage antibodies to lysozyme:
effect of coating concentration

·····□····	FDISCFVD1.3(lys)	
—◇—	FDIPs/Xh(lys)	
—■—	FDISCFVD1.3 (BSA)	
—▲—	FDIPs/Xh (BSA)	

O.D.
405nm

coating concentration (μg/ml)

Methods as described in example 4

Fig.8. Sequence around the cloning site of
fd-CAT2.

Cleavage site

```
                              Q   V   Q   L   Q        L   E   I   K   R
TAT TCT CAC AGT GCA CAG GTC CAA CTG CAG GAG CTC GAG ATC AAA CGG
            Apa LI                     PstI        Xho
```

```
GCG GCC GCA GAA ACT GTT GAA AGT etc.
Not I
```

Restriction enyme sites are shown as well as the amino acids encoded by antibody derived sequences.These are flanked at the 5' end by the gene 3 signal peptide and at the 3' end by 3 alanine residues (encoded by the Not I restriction site) and the remainder of the mature gene 3 protein.

Figure 9 Binding of pAb(D1.3) to lysozymes

Figure 10: Sequence of Fab D1.3

```
                      M  K  Y  L  L  P  T  A  A  A  G  L  L  L  P  A  A  Q  P  A
CATGCAAATTCTATTTCAAGGAGACAGTCATAATGAAATACCTATTGCCTACGGCAGCCGCTGGATTGTTATTACCTGCTGCCCAACCAG
       11        21        31        41        51        61        71        81        91


   M  A  Q  V  Q  L  Q  E  S  G  P  G  L  V  A  P  S  Q  S  L  S  I  T  C  T  V  S  G  F  S
CGATGGCCCAGGTGCAGCTGCAGGAGTCAGGACCTGGCCTGGTGGCGCCCTCACAGAGCCTGTCCATCACATGCACCGTCTCAGGGTTCT
      101       111       121       131       141       151       161       171       181


   L  T  G  Y  G  V  N  W  V  R  Q  P  P  G  K  G  L  E  W  L  G  M  I  W  G  D  G  N  T  D
CATTAACCGGCTATGGTGTAAACTGGGTTCGCCAGCCTCCAGGAAAGGGTCTGGAGTGGCTGGGAATGATTTGGGGTGATGGAAACACAG
      191       201       211       221       231       241       251       261       271


   Y  N  S  A  L  K  S  R  L  S  I  S  K  D  N  S  K  S  Q  V  F  L  K  M  N  S  L  H  T  D
TATAATTCAGCTCTCAAATCCAGACTGAGCATCAGCAAGGACAACTCCAAGAGCCAAGTTTTTCTTAAAAATGAACAGTCTGCACACTG
      281       291       301       311       321       331       341       351       361


   D  T  A  R  Y  Y  C  A  R  E  R  D  Y  R  L  D  Y  W  G  Q  G  T  T  V  T  V  S  S  A  S
ATGACACAGCCAGGTACTACTGTGCCAGAGAGAGAGATTATAGGCTTGACTACTGGGGCCAAGGCACCACGGTCACCGTCTCCTCAGCCT
      371       381       391       401       411       421       431       441       451


   T  K  G  P  S  V  F  P  L  A  P  S  S  K  S  T  S  G  G  T  A  A  L  G  C  L  V  K  D  Y
CCACCAAGGGCCCATCGGTCTTCCCCCTGGCACCCTCCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGCTGCCTGGTCAAGGACT
      461       471       481       491       501       511       521       531       541


   F  P  E  P  V  T  V  S  W  N  S  G  A  L  T  S  G  V  H  T  F  P  A  V  L  Q  S  S  G  L
ACTTCCCCGAACCGGTGACGGTGTCGTGGAACTCAGGCGCCCTGACCAGCGGCGTGCACACCTTCCCGGCTGTCCTACAGTCCTCAGGAC
      551       561       571       581       591       601       611       621       631
```

Fig. 10. cont. (1)

```
      Y  S  L  S  S  V  V  T  V  P  S  S  S  L  G  T  Q  T  Y  I  C  N  V  N  H  N  P  S  N  T
●   CTACTCCCTCAGCAGCGTGGTGACTGTGCCCTCCAGCAGCTTGGGCACCCAGACCTACATCTGCAACGTGAATCACAACCCCAGCAACA
      641       651       661       671       681       691       701       711       721
```

```
      K  V  D  K  K  V  E  P  K  S  S  *  *                                              M  K
    CCAAGGTCGACAAGAAAGTTGAGCCCAAATCTTCATAATAACCCGGGAGCTTGCATGCAAATTCTATTTCAAGGAGACAGTCATAATGAA
      731       741       751       761       771       781       791       801       811
```

```
      Y  L  L  P  T  A  A  A  G  L  L  L  P  A  A  Q  P  A  M  A  D  I  E  L  T  Q  S  P  A  S
    TACCTATTGCCTACGGCAGCCGCTGGATTGTTATTACCTGCTGCCCAACCAGCGATGGCCGACATCGAGCTCACCCAGTCTCCAGCCTC
      821       831       841       851       861       871       881       891       901
```

```
      L  S  A  S  V  G  E  T  V  T  I  T  C  R  A  S  G  N  I  H  N  Y  L  A  W  Y  Q  Q  K  Q
●   CCTTTCTGCGTCTGTGGGAGAAACTGTCACCATCACATGTCGAGCAAGTGGGAATATTCACAATTATTTAGCATGGTATCAGCAGAAACA
      911       921       931       941       951       961       971       981       991
```

```
      G  K  S  P  Q  L  L  V  Y  Y  T  T  T  L  A  D  G  V  P  S  R  F  S  G  S  G  S  G  T  Q
    GGGAAAATCTCCTCAGCTCCTGGTCTATTATACAACAACCTTAGCAGATGGTGTGCCATCAAGGTTCAGTGGCAGTGGATCAGGAACACA
      1001      1011      1021      1031      1041      1051      1061      1071      1081
```

```
      Y  S  L  K  I  N  S  L  Q  P  E  D  F  G  S  Y  Y  C  Q  H  F  W  S  T  P  R  T  F  G  G
    ATATTCTCTCAAGATCAACAGCCTGCAGCCTGAAGATTTTGGGAGTTATTACTGTCAACATTTTTGGAGTACTCCTCGGACGTTCGGTGG
      1091      1101      1111      1121      1131      1141      1151      1161      1171
```

```
      G  T  K  L  E  I  K  R  T  V  A  A  P  S  V  F  I  F  P  P  S  D  E  Q  L  K  S  G  T  A
    AGGCACCAAGCTCGAGATCAAACGGACTGTGGCTGCACCATCTGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAACTGC
      1181      1191      1201      1211      1221      1231      1241      1251      1261
```

Fig. 10. cont. (2)

```
S   V   V   C   L   L   H   N   F   Y   P   R   E   A   K   V   Q   W   K   V   D   N   A   L   Q   S   G   N   S   Q
CTCTGTTGTGTGCCTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACAGTGGAAGGTGGATAACGCCCTCCAATCGGGTAACTCCCA
     1271        1281        1291        1301        1311        1321        1331        1341        1351
```

```
E   S   V   T   E   Q   D   S   K   D   S   T   Y   S   L   S   S   T   L   T   L   S   K   A   D   Y   E   K   H   K
GGAGAGTGTCACAGAGCAGGACAGCAAGGACAGCACCTACAGCCTCAGCAGCACCCTGACGCTGAGCAAAGCAGACTACGAGAAACACAA
     1361        1371        1381        1391        1401        1411        1421        1431        1441
```
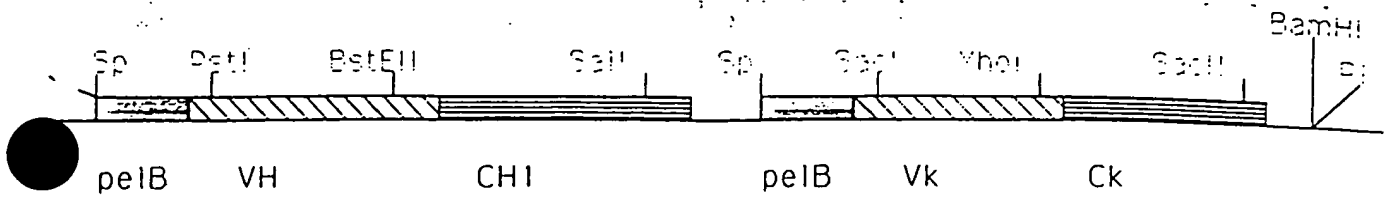
```
V   Y   A   C   E   V   T   H   Q   G   L   S   S   P   V   T   K   S   F   N   R   G   E   S   *   *
AGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGTTCGCCCGTCACAAAGAGCTTCAACCGCGGAGAGTCATAGTAAGGATCCAGCTC
     1451        1461        1471        1481        1491        1501        1511        1521        1531
```

GAATTC

FabD1.3 in
pUC19

fig.10 cont.(3).

O.D. 405nm

2

1

0

Vector
Fd ScFv (OX)
Fd VHCH1 (D1.3)
Fd Fab (D1.3)
Fd ScFv (D1.3)

Fig. 11 Comparison of lysozyme-binding by phage-Fab and phage -ScFv
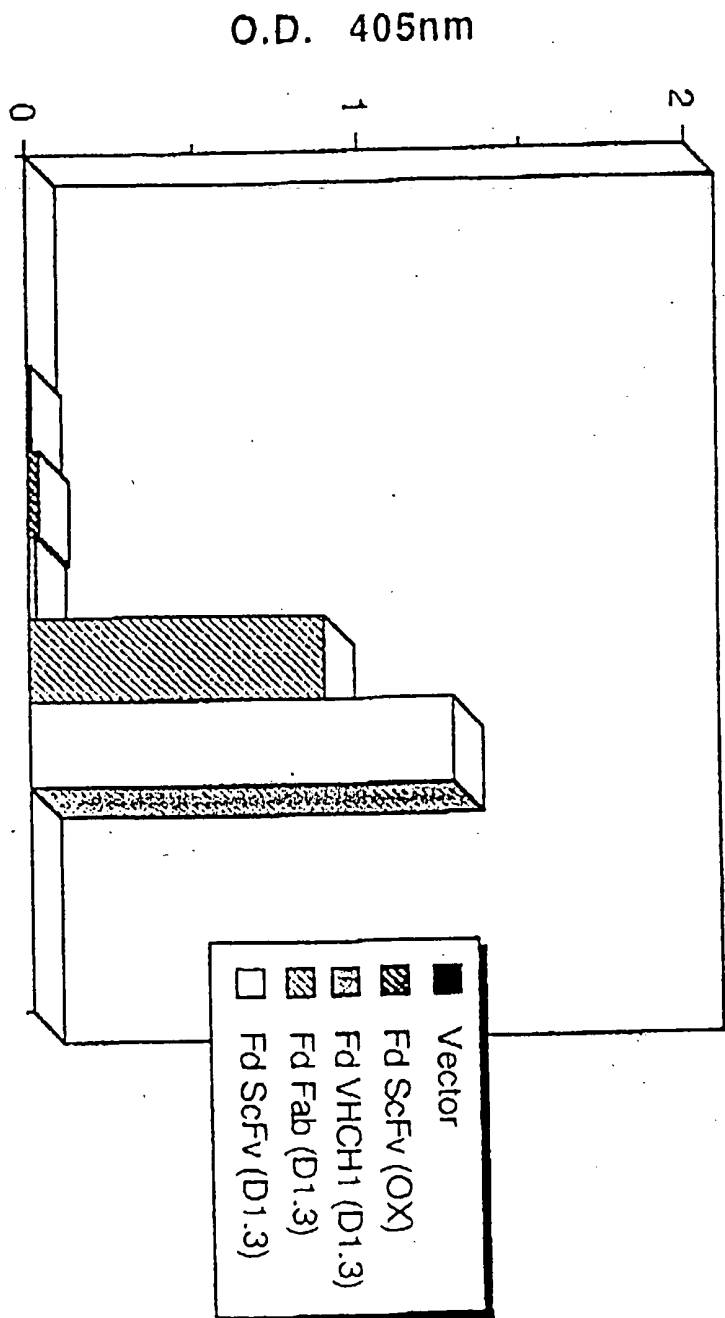
Fig. 12

B

A

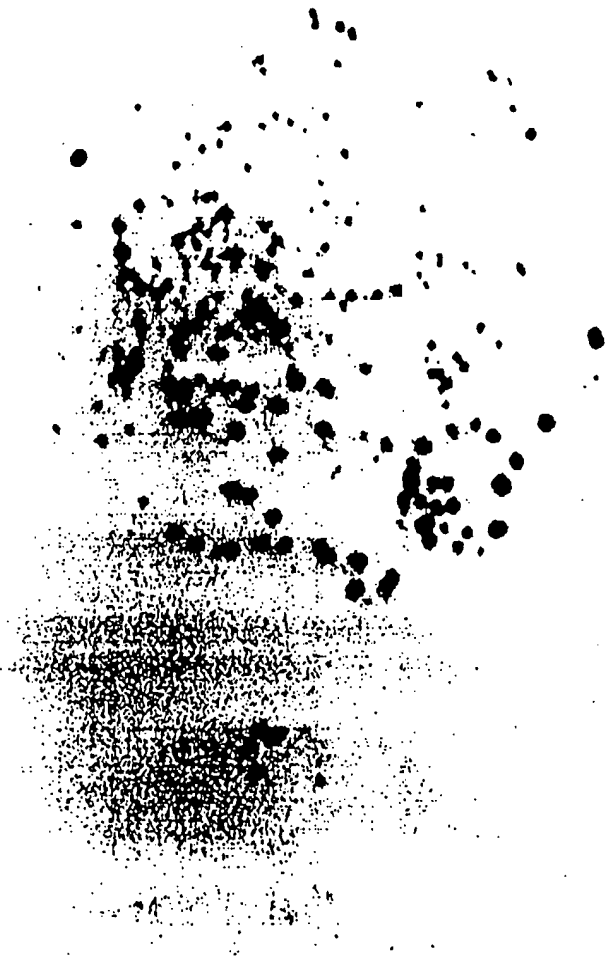## Figure 18 Sequence of pNQ11ScFv

```
 Q   V   Q   L   Q   E   S   G   G   G   L   V   Q   P   G   G
CAG GTG CAG CTG CAG GAG TCA GGA GGA GGC TTG GTA CAG CCT GGG GGT
            PstI

 S   L   R   L   S   C   A   T   S   G   F   T   F   S   N   Y
TCT CTG AGA CTC TCC TGT GCA ACT TCT GGG TTC ACC TTC AGT AAT TAC

 Y   M   G   W   V   R   Q   P   P   G   K   A   L   E   W   L
TAC ATG GGC TGG GTC CGC CAG CCT CCA GGA AAG GCA CTT GAG TGG TTG

 G   S   V   R   N   K   V   N   G   Y   T   T   E   Y   S   A
GGT TCT GTT AGA AAC AAA GTT AAT GGT TAC ACA ACA GAG TAC AGT GCA

 S   V   K   G   R   F   T   I   S   R   D   N   F   Q   S   I
TCT GTG AAG GGG CGG TTC ACC ATC TCC AGA GAT AAT TTC CAA AGC ATC

 L   Y   L   Q   I   N   T   L   R   T   E   D   S   A   T   Y
CTC TAT CTT CAA ATA AAC ACC CTG AGA ACT GAG GAC AGT GCC ACT TAT

 Y   C   A   R   G   Y   D   Y   G   A   W   F   A   Y   W   G
TAC TGT GCA AGA GGC TAT GAT TAC GGG GCC TGG TTT GCT TAC TGG GGC

 Q   G   T   L   V   T   v   s   s   g   g   g   g   s   g   g   g   g   s
CAA GGG ACC CTG GTC ACC gtc tcc tca ggtggaggcggttcaggcggaggtggctct
            BstEII

 g   g   g   g   s   d   i   E   L   T   Q   T   P   L   S   L   P   V
ggcggtggcggatcggac atc GAG CTC ACC CAA ACT CCA CTC TCC CTG CCT GTC
                       SacI

 S   L   G   D   Q   A   S   I   S   C   R   S   S   Q   S   I
AGT CTT GGA GAT CAA GCC TCC ATC TCT TGC AGA TCT AGT CAG AGC ATT

 V   H   S   N   G   N   T   Y   L   E   W   Y   L   Q   K   P
GTA CAT AGT AAT GGA AAC ACC TAT TTA GAA TGG TAC CTG CAG AAA CCA
                                               PstI

 G   Q   S   P   K   L   L   I   Y   K   V   S   N   R   F   S
GGC CAG TCT CCA AAG CTC CTG ATC TAC AAA GTT TCC AAC CGA TTT TCT

 G   V   P   D   R   F   S   G   S   G   S   G   T   D   F   T
GGG GTC CCA GAC AGG TTC AGT GGC AGT GGA TCG GGG ACA GAT TTC ACA

 L   K   I   S   R   V   E   A   E   D   L   G   V   Y   Y   C
CTC AAG ATC AGC AGA GTG GAG GCT GAG GAT CTG GGA GTT TAT TAC TGC

 F   Q   G   S   H   V   P   Y   T   F   G   G   G   T   K   L
TTT CAA GGT TCA CAT GTT CCG TAC ACG TTC GGA GGG GGG ACC AAG CTC

 E   I   K   R
GAG ATC AAA CGG
XhoI
```

Restriction sites refered to in the text are shown underlined. The sequence contributed by the linker is shown in lower case.

Fig. 15. Sequence surrounding phoA insertion in fd-phoA1

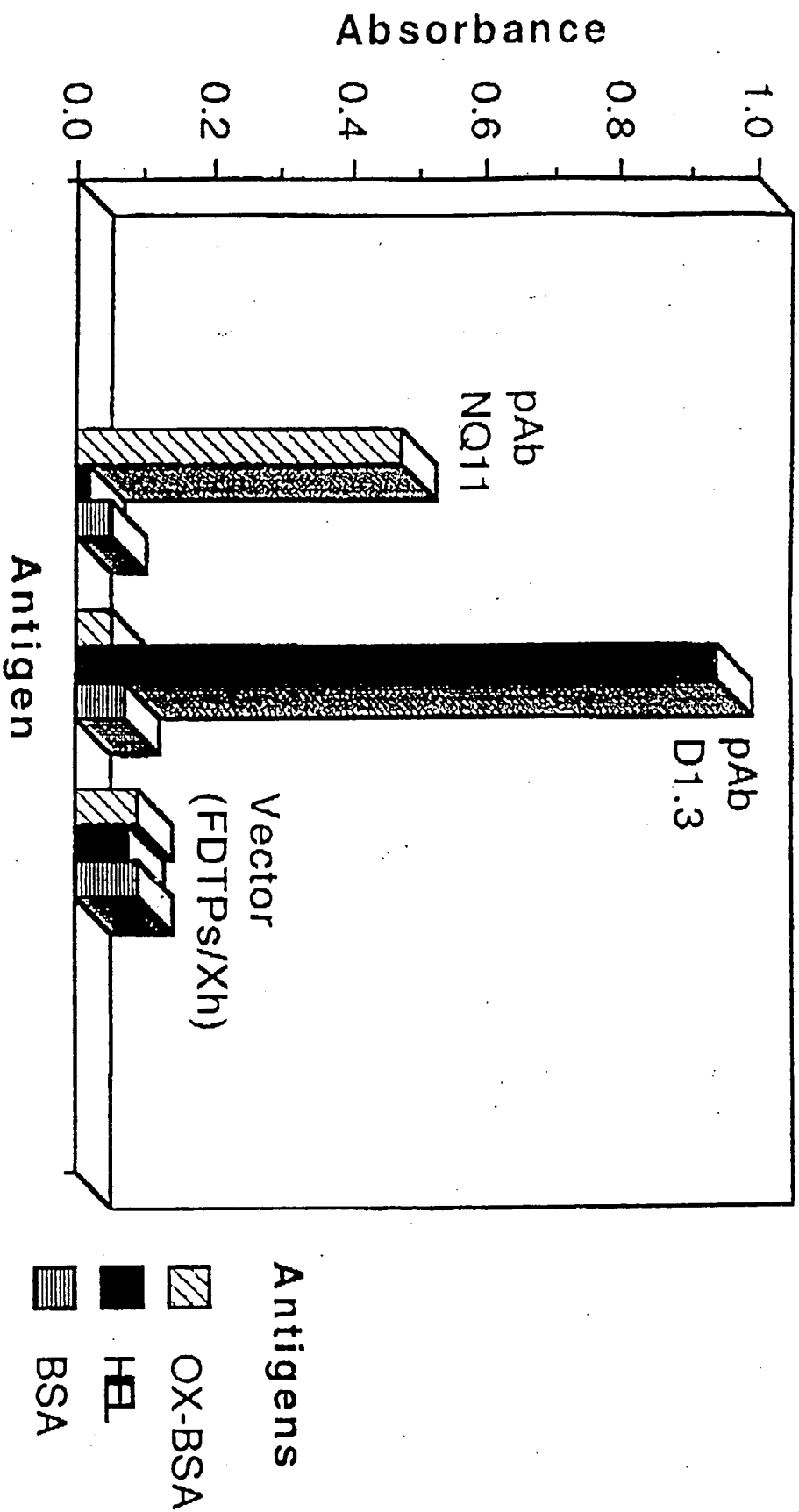SEQUENCE AT 5' END OF phoA INSERTION IN fd-phoA1

Signal peptide
cleavage site

               ▼   R   T   P   E   M   P   V   L

TCT CAC A<u>GT GCA C</u>AA ACT GTT GAA CGG ACA CCA GAA ATG CCT GTT CTG
        ApaL1

SEQUENCE AT 3' END OF phoA INSERTION IN fd-phoA1

   K   A   A   L   G   L   K

AAA GCC GCT CTG GGG CTG AAA <u>GCG GCC GC</u>A GAA ACT GTT GAA AGT etc.
                                       NotI

The restriction sites used for cloning are shown as well as the as the amino acids encoded by phoA around the insertion site. In this example, the first five amino acids of the mature fusion will actually come from gene 3.
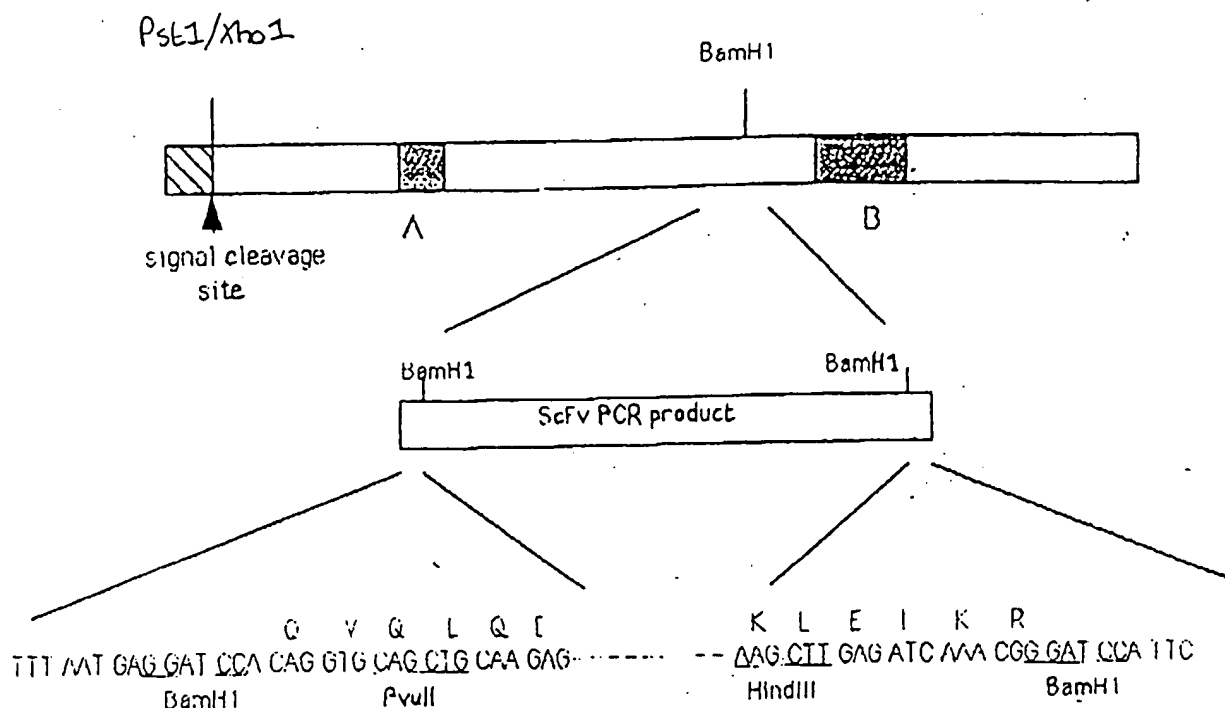
Figure14.Binding of pAbs to specific antigens

igure 16. Structure of gene 3

1)

PstI/XhoI

BamH1

signal cleavage
site

A   B

BamH1   BamH1

ScFv PCR product

Q V Q L Q E   K L E I K R
TTT AAT GAG GAT CCA CAG GTG CAG CTG CAA GAG - - - - - - - - AAG CTT GAG ATC AAA CGG GAT CCA TTC
     BamH1   PvuII   HindIII   BamH1

2)   A   (1834) 5'   GAG GGT GGT GGC TCT
                    "   "   "C "   "
                    "   "   "C "   "
                    "   "   "C "   ACT 3' (1839)

       B   (2284) 5' -   GGC GGC GGC TCT
                    .   GGT GGT GGT "
                    .   " GGC GGC "
                   GAG "   " GGC "
                    "   "   " GGT "
                    "   "   " GGC "
                    "   "   " GGT "
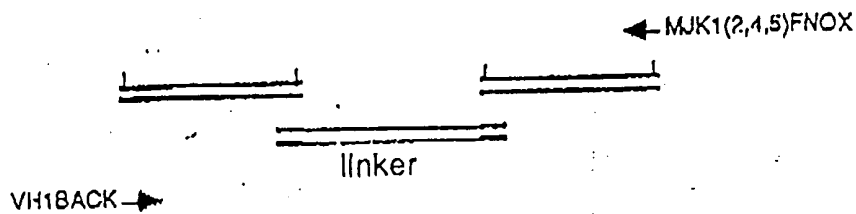   "   "   " GGC " 3' (2379)

verse complement of mutagenic
go G3Bamlink   T
                         5'   CAG GGT GGC GGA TCC

                                    1
                         GAG GGT GGC GG 3'
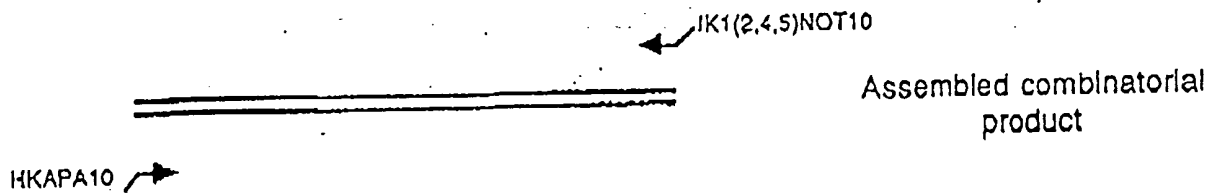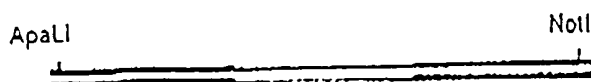
ring is according to Beck et al (1978, supra).

VH1BACK→

VK2BACK→

←VHIFOR-2

←MJK1(2,4,5)FNOX

Fig.17

↓ PRIMARY PCR FROM cDNA

Heavy   Kappa

↓ MIX PRODUCTS WITH LINKER DNA
(MADE BY PCR)

←MJK1(2,4,5)FNOX

Primary PCR
products

linker

VH1BACK→

↓ SECOND ASSEMBLY PCR
USING 'OUTER' PRIMERS

←JK1(2,4,5)NOT10

Assembled combinatorial
product

HKAPA10→

↓ PULL-THROUGH (tagged primers)

ApaLI   NotI

↓ DIGEST AND CLONE

a b c M1 M2
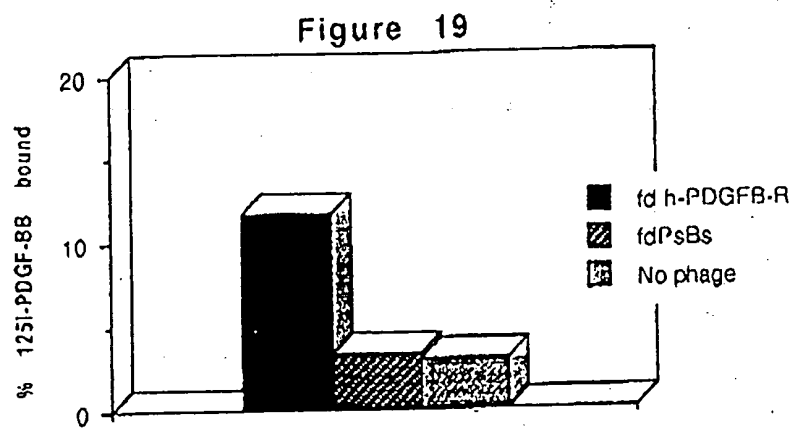
Fig. 18

Figure 19

Figure 20 Displacement by unlabelled PDGF

Figure 21 PGDF displacement vector binding deducted

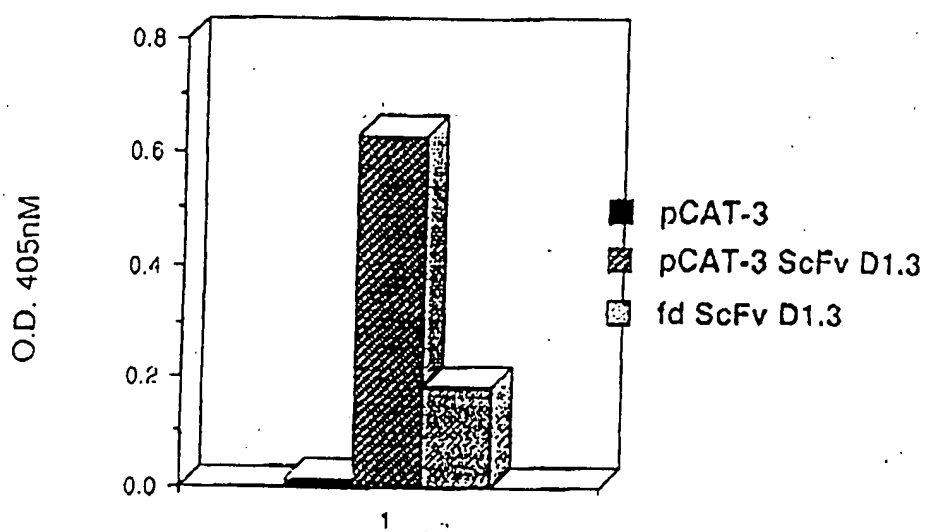[125I PDGF bound] pM

Unlabelled PDGF nM

Figure 22: Elisa of lysozyme binding by pCAT-3 ScFv D1.3 phagemid in comparison with pCAT-3 vector (both rescued by M13KO7) and fdCAT2 ScFvD1.3 as described in example 17. ELISA was performed as described in example 6 with modifications detailed in example 17.

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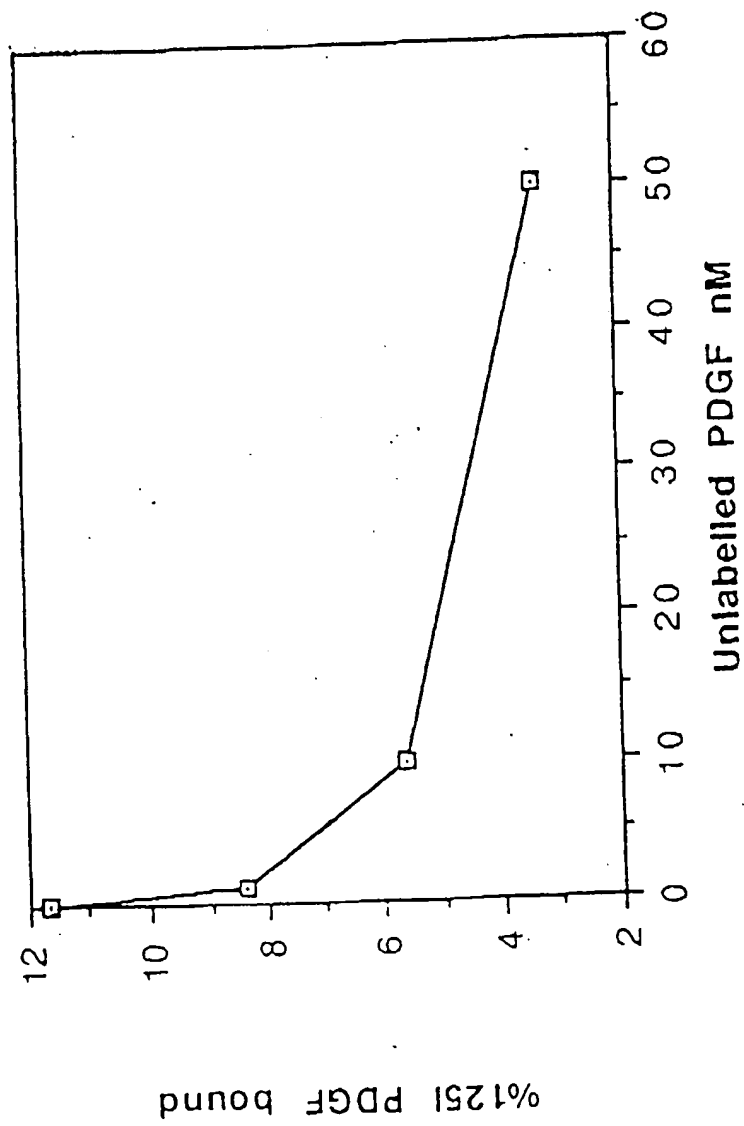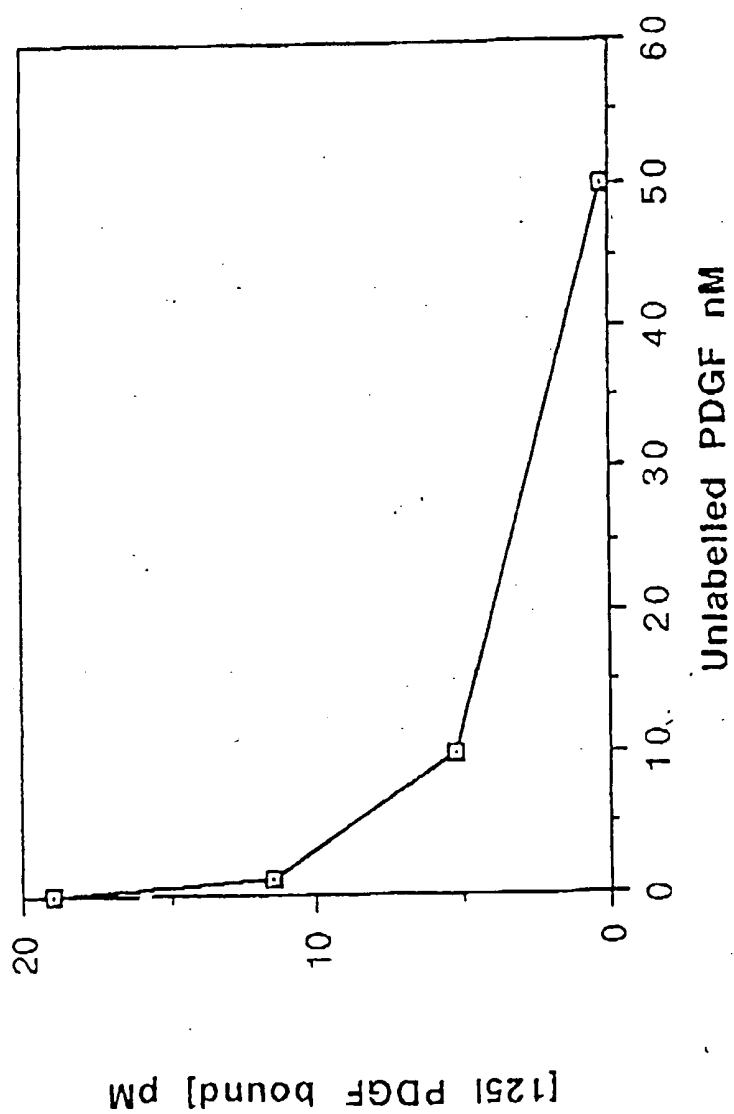
Specification for British Patent Application

CASE No: COB/CP9500687

APPLICATION No:	9104744
DATE FILED:	/3/91
PRIORITY:	

APPLICANTS: O'BRIEN, Caroline Jane
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Avon

TITLE: BINDING SUBSTANCES

INVENTORS: CHISWELL, David John
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London N15

DERIVATION OF RIGHT TO APPLY:

PRIORITY CLAIM: FIRST FILING

BINDING SUBSTANCES

The present invention relates to binding substances. The present invention also relates to methods for the production of binding substances eg binding molecules and to the biological binding molecules produced by these methods. The present invention also relates to: a) the production of antibodies, receptor molecules and fragments and derivatives of these antibodies and receptor molecules; b) viruses encoding the above identified molecules, which viruses have the ability to present said molecules at their surfaces; c) packages comprising a virus and an above identified molecule presented at the viral surface; and d) screening techniques utilising the unique properties of these packages.

Owing to their high specificity for a given antigen, the advent of monoclonal antibodies (Kohler, G. and Milstein C; 1975 Nature $\underline{256}$: 495) represented a significant technical break-through with important consequences both scientifically and commercially.

Monoclonal antibodies are made by establishing an immortal mammalian cell line which is derived from a single immunoglobulin producing cell secreting one form of a biologically functional antibody molecule with a particular specificity. Because the antibody-secreting mammalian cell line is immortal, the characteristics of the antibody are reproducible from batch to batch. The key properties of monoclonal antibodies are their specificity for a particular antigen and the reproducibility with which they can be manufactured.

Structurally, the simplest antibody (IgG) comprises four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulphide bonds (see figure 1). Each chain has a constant region (C) and a variable region (V). The antibody has two arms (the Fab region) each of which has a V_L and a V_H region associated with each other. It is this pair of V regions (V_L and V_H) that differ

from one antibody to another, and which together are responsible for recognising the antigen. In even more detail, each V region is made up from three complementarity determining regions (CDR) separated by four framework regions (FR). The CDR's are the most variable part of the variable regions, and they perform the critical antigen binding function. The CDR regions are derived from many potential germ line sequences via a complex process involving recombination, mutation and selection.

It has been shown that the function of binding antigens can be performed by fragments of a whole antibody. Binding fragments are the F_V fragment which comprises the V_L and V_H of a single arm of the antibody, and the dAb fragment (Ward, E.S. et al., Nature $\underline{341}$, 544-546 (1989); which consists of a single heavy chain variable domain (V_H).

Although the F_V fragment is coded for by separate genes, it has proved possible to construct a linker that enables them to be made as a single protein chain (known as single chain F_V (scFv); Bird, R.E. et al., Science $\underline{423}$, 423-426 (1988) Huston, J.S. et al., Proc. Natl. Acad. Sci., USA $\underline{85}$, 5879-5883 (1988)) by recombinant methods.

Whilst monoclonal antibodies, their fragments and derivatives have been enormously advantageous, there are nevertheless a number of limitations associated with them.

Firstly, the therapeutic applications of monoclonal antibodies produced by human immortal cell lines holds great promise for the treatment of a wide range of diseases (Clinical Applications of Monoclonal Antibodies. Edited by E. S. Lennox. British Medical Bulletin 1984. Publishers Churchill Livingstone). Unfortunately, immortal antibody-producing human cell lines are very difficult to establish and they give low yields of antibody (approximately 1 µg/ml). In contrast, equivalent rodent cell lines yield high amounts of antibody (approximately 100 µg/ml). However, the repeated administration of these foreign rodent proteins to humans can lead to harmful hypersensitivity reactions. In the main therefore, these

rodent-derived monoclonal antibodies have limited therapeutic use.

Secondly, a key aspect in the isolation of monoclonal antibodies is how many different antibody producing cells with different specificities, can be sampled, compared to how many need to be sampled in order to isolate a cell producing antibody with the desired specificity characteristics (Milstein, C., Royal Soc. Croonian Lecture, Proc. R. Soc. London B. $\underline{239}$; 1-16, (1990)). For example, the number of different specificities expressed at any one time by lymphocytes of the murine immune system is thought to be approximately 10^7 and this is only a small proportion of the potential repertoire of specificities. However, during the isolation of a typical antibody producing cell with a desired specificity, the investigator is only able to sample 10^3 to 10^4 individual specificities. The problem is worse in the human, where one has approximately 10^{12} lymphocyte specificities, with the limitation on sampling of 10^3 or 10^4 remaining.

This problem has been alleviated to some extent in laboratory animals by the use of immunisation regimes. Thus, where one wants to produce monoclonal antibodies having a specificity against a particular epitope, an animal is immunised with an immunogen expressing that epitope. The animal will then mount an immune response against the immunogen and there will be a proliferation of lymphocytes which have specificity against the epitope. Owing to this proliferation of lymphocytes with the desired specificity, it becomes easier to detect them in the sampling procedure. However, this approach is not successful in all cases, as a suitable immunogen may not be available. Furthermore, where one wants to produce human monoclonal antibodies (eg for therapeutic administration as previously discussed) such an approach is not practically or ethically feasible.

In the last few years, these problems have in part,

been addressed by the application of recombinant DNA methods to the isolation and production of antigen binding fragments of an antibody molecule in bacteria such as E.coli. Furthermore, the use of polymerase chain reaction (PCR) amplification (Saiki, R.K., et al., Science 239, 4387-491 (1988)) to isolate antibody producing sequences from cells and organs, has great potential for speeding up the timescale under which specificities can be isolated. Amplified V_H and V_L genes are cloned directly into vectors for expression in bacteria or mammalian cells (Orlandi, R., et al., 1989, Proc. Natl. Acad. Sci., USA 86, 3833-3837; Ward, E.S., et al., 1989 supra; Larrick, J.W., et al., 1989, Biochem. Biophys. Res. Commun. 160, 1250-1255; Sastry, L. et al., 1989, Proc. Natl. Acad. Sci., USA., 86, 5728-5732). Conversely, some of these techniques can exacerbate the screening problems. For example, large separate heavy and light chain libraries have been produced from immunized mice and combined together in a random combinatorial manner prior to screening (Huse, W.D. et al., 1989, Science 246, 1275-1281). Crucially however, the information held within each cell, namely the specific combination of one light chain with one heavy chain, is lost. This loses most, if not all, of the advantage gained by using immunization protocols in the animal. Currently, only libraries derived from single heavy chain variable domains (dAbs; Ward, E.S., et al., 1989, supra.) do not suffer this drawback, but because not all antibody heavy chain variable regions are capable of binding antigen, more have to be screened.

In addition, the problem of directly screening many different specificities in prokaryotes remains to be solved.

Thus, there is a need for a screening system which ameloriates or overcome one or more of the above or other problems. The ideal system would allow the sampling of very large numbers of specificities (eg of the order of 10^6 and higher) rapid sorting at each cloning round, and rapid

transfer of the genetic material coding for the binding molecule from one stage of the production process, to the next stage.

The most attractive candidates for this type of screening, would be prokaryotic organisms (because they grow quickly, are relatively simple to manipulate and because large numbers of clones can be created) which express and retain antibody on their surface. It has already been shown that antibody fragments can be secreted through bacterial membranes with the appropriate signal peptide (Skerra, A., and Pluckthun, A., 1988, Science 240, 1038-1040; Better, M. et al., 1988, Science 240, 1041-1043). However, it has not been shown how an antibody or antibody fragment can be held on the bacterial cell surface in a configuration which allows efficient sampling of its antigen binding properties. In large part, this is because the bacterial surface is a complex structure, and in the gram-negative organisms there is an outer wall which further complicates the position.

Bacteriophage make attractive candidates because in general their surface is a much simpler structure, they can be grown easily in large numbers, are amenable to the practical handling involved in many potential mass screening programmes and they carry genetic information for their own synthesis within a small, simple package. The difficulty has been to practically solve the problem of how to use bacteriophages in this manner. For example, a Genex Corporation patent application number PCT/US88/00716 has proposed that the bacteriophage lambda would be a suitable vehicle for the expression of antibody molecules, but no proposals provide a teaching which enables the general idea to be carried out. For example PCT/US88/00716 does not demonstrate that any sequences: a) have been expressed as a fusion with gene V; b) have been expressed on the surface of lambda; and c) retain biological activity. Furthermore there is no teaching on how to screen for suitable fusions.

The problem of how to use bacteriophages is in fact a

difficult one. The antibody molecule must be inserted into the phage in such a way that the integrity of the phage coat is not undermined, and the antibody itself should be biologically active. Thus the antibody should fold efficiently and correctly and be presented for antigen binding. However, solving the problem for antibody molecules and fragments would also provide a general method for the screening of many receptor molecules.

Surprisingly, the applicants have been able to construct a bacteriophage that expresses and presents on its surface large binding molecules (eg large biologically functional antibody molecules) and which remains intact and infectious. The applicants have called the structure which comprises a virus particle and a binding molecule presented at the viral surface a 'package'. Where the binding molecule is an antibody (or a fragment or derivative of an antibody), the applicants call the package a phage antibody. However, except where the context demands otherwise, where the term phage antibody is used generally it should also be interpreted as referring to any package comprising a virus particle and a binding molecule presented at the viral surface. Phage-antibodies (pAbs) are likely to find a range of applications in screening antibody V-genes encoding antigen binding activities. For example, pAbs could be used in cloning and rescue of hybridomas (Orlandi, R., et al (1989) PNAS 86 p3833-3837), and in the screening of large combinatorial libraries (such as found in Huse, W.D. et al., 1989, Science 246, 1275-1281) In particular, rounds of selection using pAbs may help in rescuing the higher affinity antibodies from the latter libraries. It may be preferable to screen small libraries derived from antigen-selected cells (Casali, P., et al., (1986) Science 234 p476-479) to rescue the original VH/VL pairs comprising the V region of an antibody. The use of pAbs may also allow the construction of entirely synthetic antibodies. For example, V-gene repertoires could be made in vitro by combining unrearranged V genes, with D and J segments. Libraries of

pAbs could then be selected by binding to antigen, hypermutated in the antigen-binding loops in vitro and subjected to further rounds of selection and mutagenesis.

5 The demonstration that a functional antigen-binding site can be expressed on the surface ·of phage, has implications beyond the construction of novel antibodies. For example, if other protein domains can be expressed at the surface of a phage, phage vectors could be used to clone and select genes by the binding properties of the expressed

10 protein. Furthermore, endless variants of proteins, including epitope libraries built into the surface of the protein, could be made and readily selected for binding activities. In effect other protein architectures might serve as "nouvelle" antibodies. One class of molecules that

15 could be useful in this type of application are receptors. For example, a specific receptor could be expressed on the surface of the phage such that it would bind its ligand. The receptor could then be modified by, for example, in vitro mutagenesis and variants having higher binding

20 affinity for the ligand selected. The screening may be carried out according to one or more of the formats described below with reference to figure 2. Figure 2 refers particularly to phage antibodies. In the situation discussed above the phage antibody is replaced with a phage

25 receptor and the antigen with a ligand 1.

Alternatively, the receptor-phage could be used as the basis of a rapid screening system for the binding of ligands, altered ligands, or potential drug candidates. The advantages of this system of simple cloning, convenient

30 expression, standard reagents and easy handling makes the drug screening application particularly attractive. In the context of this discussion, receptor means a molecule that binds a specific, or group of specific, ligand(s); the natural receptor could be expressed on the surface of a

35 population of cells, it could be the extracellular domain of such a molecule (whether such a form exists naturally or not), or it could be a soluble molecule performing a natural

binding function in the plasma or within a cell or organ.

Although throughout this application the applicant discuss the possibility of screening for higher affinity variants of phage-antibodies, they recognise that in some applications, for example low affinity chromatography (Ohlson, S. et al Anal. Biochem. 169, p204-208 (1988)), it may be desirable to isolate lower affinity variants.

The system of expressing binding molecules on the phage surface could also be used as a primary cloning system. For example, a cDNA library could be constructed and inserted into the bacteriophage and this library screened for the ability to bind a ligand. The ligand/binding molecule combination could include receptor/ligand, enzyme/substrate (or analogue), nucleic acid binding protein/nucleic acid etc. This could be a preferred method to isolate a clone of either molecule in the pair, if the other molecule was available.

Targeted gene transfer

A useful and novel set of applications makes use of the binding protein on the phage to target the phage genome to a particular cell or group of cells. For example, a phage-antibody specific for a cell surface receptor could be used to bind to the target cell surface. The phage could then be internalised, either through the action of the receptor itself or as the result of another event (eg. an electrical discharge such as in the technique of electroporation). The phage genome would then be expressed if the relevent control signals (for transcription and translation and possibly replication) were present. This would be particularly useful if the genome contained a sequence whose expression was desired in the target cell (along with the appropriate expression control sequences). A useful sequence might confer antibiotic resistance to the recipient cell or label the cell by the expression of its product (eg. if the sequence expressed a detectable gene product such as a

luciferase, see White, M, et al, Techniques 2(4), p194-201 (1990)), or confer a particular property on the target cell (eg. if the target cell was a tumour cell and the new sequence directed the expression of a tumour suppressing gene), or express an antisense construct designed to turn off a gene or set of genes in the target cell, or express a gene or gene product designed to be toxic to the target cell.

This technique of "targeted gene transfer" has a number of uses in research and also in therapy and diagnostics. For example, gene therapy often aims to target the replacement gene to a specific cell type that is deficient in its activity; targeted pAbs provide an answer to this problem. In diagnostics, phage specific for particular bacteria or groups of bacteria have been used to target marker genes, eg. luciferase, to the bacterial host (sec, for example, Ulitzer, S., and Kuhn, J., EPA 85303913.9). If the host range of the phage is appropriate, only those bacteria that are being tested for, will be infected by the phage, express the luciferase gene and be detected by the light they emit. This system has been used to detect the presence of Salmonella. One major problem with this approach is the initial isolation of a bacteriophage with the correct host range and then cloning a luciferase gene cassette into that phage, such that it is functional. The pAb system allows the luciferase cassette to be cloned into a well characterised system (filamentous phage) and allows simple selection of an appropriate host range, by modifying the antibody (or other binding molecule) specificity the pAb contains.

The applicant has also shown that enzymes can be expressed on the phage surface. Useful applications of this invention include the cloning of enzyme coding genes, or the design and selection of mutant enzymes with enhanced properties on particular substrates. For example, conditions can be used whereby the enzyme (or modified enzyme) binds a particular substrate, product or

intermediate (or analogues of them) to identify phage from a library containing a desired activity or by subjecting phage already expressing the enzyme, to in vitro mutagenesis, followed by selection of those variants with a desired level

5   of binding and/or catalysis.

The present applicants have also been able to develop novel screening systems and assay formats which depend on the unique properties of these packages eg phage antibodies.

The present invention provides a method for producing a

10   package which method comprises the steps of:

a)   inserting a nucleotide sequence encoding the binding molecule within a viral genome;

b)   culturing the virus containing said nucleotide sequence, so that said binding molecule is expressed by

15   the virus presented at its surface.

The present invention also provides a method for producing a binding molecule specific for a particular epitope which comprises producing a package as described above and the additional step of screening for said binding

20   molecule by binding of said molecule to said epitope. The method may comprise one or more of the additional steps of: i) separating the package from the epitope; ii) recovering said package; and iii) using the inserted nucleotide sequence in a recombinant system to produce the binding

25   molecule separate from virus. The screening step may isolate the nucleotide sequence encoding the binding molecule of desired specificity, by virtue of said binding molecule being expressed in association with the surface of the virus.

30   In the above methods, the binding molecule may be an antibody, or a fragment or derivative of an antibody. Alternatively, the binding molecule may be an enzyme or receptor and fragments/derivatives of any such enzymes or receptors.

35   In the above methods, the virus may be a filamentous F-specific bacteriophage. The filamentous F-specific bacteriophage may be fd. In particular, it may be a

tetracycline resistant version of fd known as fd-tet. The nucleotide sequence may be inserted within the gene III region of fd. The sequence may be inserted after the signal sequence of gene III, preferably after amino acid+1 of the mature protein. The site for insertion may be flanked by short sequences corresponding to sequences which occur at each end of the DNA to be inserted. For example, the protein domain is an immunoglobulin domain, the insertion site in the phage may be flanked by nucleotide sequences which code for the first five amino acids and the last five amino acids of the Ig domain. Such flanking nucleotide sequences are shown in figure 4(2) B and C, wherein the site-flanking nucleotide sequences encode amino acid sequences QVQLQ and VTVSS which occur at either end of the V_H domain, or QVQLQ and LEIKR which occur at either end of the F_V (combined V_H + V_L) domain. Each of these sequences flanking the insertion site may include a suitable cleavage site, as shown in Fig 4.

Alternatively, the flanking nucleotide sequences shown in figure 4(2) B and C as described above, may be used to flank the insertion site for any DNA to be inserted, whether or not that DNA codes an immunoglobulin.

In the above methods the nucleotide sequences inserted within the viral genome may be derived from eg mammalian spleen cells or peripheral blood lymphocytes. The mammal may be immunised or non-immunised. Alternatively, the nucleotide sequence may be derived by the in vitro mutagenesis of an existing antibody coding sequence. The phage particle presenting said binding molecule may remain intact and infectious.

As previously mentioned, the present invention also provides novel screening systems and assay formats. In these systems and formats the gene sequence encoding the binding molecule (eg the antibody) of desired specificity is separated from the general population having a range of specificities by the fact of its binding to a specific target (eg the antigen or epitope).

Thus, the present invention provides a method of screening a population of phage antibodies (where the binding molecule is an antibody) for a phage antibody with a desired specificity, which comprises contacting said

5   population of phage antibodies with a desired epitope and separating phage antibody which binds to said epitope, from said epitope.   The means for separating any binding phage antibodies may be varied in order to obtain binding phage antibodies with different binding affinities for said

10   epitope.

Alternatively, in order to obtain high affinity phage antibodies the epitope may be presented to the population of phage antibodies already with a binding member for said epitope bound thereto, in which case, phage antibodies with

15   a higher binding affinity for said epitope than said bound binding member will displace said bound binding member.   The high affinity phage antibodies can then be separated from said epitope.

Separation of phage antibodies from said epitope may be

20   achieved by eg elution techniques well known in the art, infection of suitable bacteria etc.

The present invention also provides packages as defined above and binding molecules (eg antibodies, enzymes, receptors; fragments and derivatives thereof), obtainable by

25   use of any of the above defined methods, systems and formats.

The applicants have chosen the filamentous F- specific bacteriophages as an example of the type of phage that could provide a vehicle for the expression of antibodies and

30   antibody fragments and derivatives on their surface and facilitate subsequent screening and manipulation.

The F-specific phages (eg fl, fd and M13) have evolved a method of propagation which does not kill the host cell and they are used commonly as vehicles for recombinant DNA

35   (Kornberg, A., DNA Replication, W.H. Freeman and Co., San Francisco, 1980).   The single stranded DNA genome (approximately 6.4 Kb) is extruded through the bacterial

membrane where it sequesters capsid sub-units, to produce mature virions. These virions are 6 nm in diameter, 1μm in length and each contain approximately 2,800 molecules of the major coat protein encoded by viral gene VIII and four molecules of the adsorption molecule encoded by viral gene III. The latter is located at one end of the virion. The structure has been reviewed by Webster et al., 1978 in The Single Stranded DNA Phages, 557-569, Cold Spring Harbor Laboratory Press. The gene III product is involved in the binding of the phage to the bacterial F-pilus.

Although these phages do not kill their host during normal replication, disruption of some of their genes can lead to cell death (Kornberg, A., 1980 supra.) This places some restraint on their use. The applicants have recognized that gene III of phage fd is an attractive possibility for the insertion of biologically active foreign sequences. The protein itself is only a minor component of the phage coat and disruption of the gene does not lead to cell death (Smith, G. 1988, Virology 167: 156-165). Furthermore, it is possible to insert some foreign sequences (with no biological function) into various positions within this gene (Smith, G., 1985 Science 228: 1315-1317., Parmley, S.F. and Smith, G.P Gene: 73 (1988) p. 305-318., and de la Cruz, V.F., et al., 1988, J. Biol. Chem., 263: 4318-4322). In these cases, although the infectivity of the virion was disrupted, the inserted sequences could be detected on the phage surface.

The protein encoded by gene III has several domains (Pratt, D., et al., 1969 Virology 39:42-53., Grant, R.A., et al., 1981, J. Biol. Chem. 256: 539-546 and Armstrong, J., et al., FEBS Lett. 135: 167-172 1981.) including: i) a signal sequence that directs the protein to the cell membrane and which is then cleaved off; ii) a domain that anchors the mature protein into the bacterial cell membrane (and also the phage coat); and iii) a domain that specifically binds to the phage receptor the F-pilus of the host bacterium. Short sequences derived from protein molecules have been

inserted into two places within the mature molecule (Smith, G., 1985 supra., and Parmley, S.F. and Smith, G.P., 1988 supra.) into an inter-domain region and also between amino acids 2 and 3 at the N-terminus. The insertion sites at the

5 N-terminus were more successful in maintaining the structural integrity of the gene III protein and displaying the peptides on the surface of the phage. By use of specific antisera, the peptides inserted into this position were shown to be on the surface of the phage. These authors

10 were also able to purify the phage using this property. However, the peptides expressed by the phage, did not possess measurable biological functions of their own.

Retaining the biological function of a molecule when it is expressed in a radically different context to its natural

15 state is difficult. The demands on the structure of the molecule are heavy. In contrast, retaining the ability to be bound by specific antisera is a passive process which imposes far less rigorous demands on the structure of the molecule. For example, it is the rule rather than the

20 exception that polyclonal antisera will recognise totally denatured, and biologically inactive, proteins on Western blots (see for example, Harlow, E. and Lane, D., Antibodies, a Laboratory Manual, Cold Spring Harbor Laboratory Press 1988). Therefore, the insertion of peptides into a region

25 that allows their structure to be probed with antisera teaches only that the region allows the inserted sequences to be exposed and does not teach that the region is suitable for the insertion of large sequences with demanding biological function.

30 This experience with Western blots is a graphic practical demonstration which shows that retaining the ability to be bound by specific antisera imposes far less rigorous demands on the structure of a molecule, than does the retention of a biological function.

35 The applicants have investigated the possibility of inserting biologically active antibody fragments into the gene III region of fd to create a large fusion protein. As

is apparent from the previous discussion, this approach makes onerous demands on the functionality of the fusion protein. The insertion is large, 100-200 amino acids; the antibody derived domain must fold efficiently and correctly to retain antigen-binding; and most of the functions of gene III must be retained. The applicants approach to the construction of the fusion molecule was designed to minimise the risk of disrupting these functions. The initial vector used was fd-tet (Zacher, A.N., et al., 1980, Gene $\underline{9}$, 127-140) a tetracycline resistant version of fd bacteriophage that can be propagated as a plasmid that confers tetracycline resistance to the infected E.coli host. The applicants chose to insert after the signal sequence of the fd gene III protein for several reasons. In particular, the applicants chose to insert after amino acid 1 of the mature protein to retain the context for the signal peptidase cleavage. To retain the structure and function of gene III itself, the majority of the original amino acids are synthesized after the inserted immunoglobulin sequences. The inserted immunoglobulin sequences were designed to include residues from the switch region that links V_H-V_L to C_H1-C_L (Lesk, A., and Chothia, C., Nature 335, 188-190, 1988).

Surprisingly, by manipulating gene III of bacteriophage fd, the present applicants have been able to construct a bacteriophage that expresses on its surface large biologically functional antibody molecules and which remains intact and infectious. Furthermore, the phages bearing antibodies of the correct specificity, can be selected from a background where the majority of phages do no show this specificity.

The population of antibody molecules inserted into the phage can be derived from a variety of sources. For example, immunised or non-immunised rodents or humans, and from organs such as spleen and peripheral blood lymphocytes. The coding sequences are derived from these sources by techniques familiar to those skilled in the art (Orlandi,

R., et al., 1989 supra; Larrick, J.W., et
al., 1989 supra; Chiang, Y.L., et al., 1989 Bio Techniques
7, p. 360-366; Ward, E.S, et al., 1989 supra; Sastry, L., et
al., 1989 supra.) Each individual phage antibody in the
resulting library of phage antibodies will express antibody
derived fragments that are monoclonal with respect to its
antigen-binding characteristics.

The disclosure made by the present applicants is
important and provides a significant breakthrough in the
technology relating to the production of biological binding
molecules, their fragments and derivatives by the use of
recombinant methods.

In standard recombinant techniques for the production
of antibodies, an expression vector containing sequences
coding for the antibody polypeptide chains is used to
transform eg E.coli. The antibody polypeptides are
expressed and detected by use of standard screening systems.
When the screen detects an antibody polypeptide of the
desired specificity, one has to return to the particular
transformed E.coli expressing the desired antibody
polypeptide. Furthermore, the vector containing the coding
sequence for the desired antibody polypeptide then has to be
isolated for use from E.coli in the further processing
steps.

In the present invention however, the desired antibody
polypeptide when expressed, is already packaged with its
gene coding sequence. This means that when the screen
detects an antibody polypeptide of desired specificity,
there is no need to return to the original culture for
isolation of that sequence.

Because the phage antibody is a novel structure that
contains an antibody of monoclonal antigen-binding
specificity on the surface of a relatively simple structure
also containing the genetic information encoding its
function, phage antibodies that bind antigen can be
recovered very efficiently by either eluting off (eg using
diethylamine, high salt etc) and infecting suitable

bacterial or by denaturing the structure and specifically amplifying the antibody encoding sequences using PCR. That is, there is no necessity to refer back to the original bacterial clone that gave rise to the phage antibody.

5   Individual phage antibodies expressing the desired antigen-binding specificity can be isolated from the complex library using the conventional screening techniques (eg as described in Harlow, E., and Lane, D., 1988, supra). One example is illustrated in figure 2(i). This shows antigen

10   (ag) bound to a solid surface (s). The population of phage antibodies is then passed over the antigen, and those individuals p that bind are retained after washing, and optionally detected with detection system d. One possible detection system based upon anti-fd antisera is illustrated

15   below in example 4. Since the bound phage antibody can be amplified using for example PCR or bacterial infection, it is also possible to rescue the desired specificity even when insufficient individuals are bound to allow detection via conventional techniques.

20   The efficiency of this screening procedure for phage antibodies and the ability to create very large libraries means that the immunisation techniques developed to increase the proportion of screened cells producing antibodies of interest will not be an absolute requirement. The technique

25   allows the rapid isolation of antigen-binding specificities, including those that would be difficult or even unobtainable by conventional techniques, for example, catalytic or anti-idiotypic antibodies. Removal of the animal altogether is now possible once a complete library of the immune

30   repertoire has been constructed.

Affinity Maturation Screening Formats

The applicants have also devised a series of novel screening techniques that are practicable only because of the unique properties of phage antibodies. The general

35   outline of some screening procedures is illustrated in figure 2.

The population/library of phage antibodies to be

screened could be generated from immunised or other animals; or be created in vitro by mutagenising pre-existing phageantibodies (using techniques well-known in the art such as oligonucleotide directed mutagenesis (Sambrook, J., et al., 1989 Molecular Cloning a Laboratory Manual, Cold Spring Harbor Laboratory Press). This population can be screened in one or more of the formats described below with reference to figure 2, to derive those individual phage antibodies whose antigen binding properties are different from sample c. Examples of the possible screening formats are:

Binding/Elution

Referring to figure 2(i) population p binds to antigen ag fixed to a solid support s. If samples of bound population p are removed under increasingly stringent conditions, the binding affinity represented in each sample will increase. Conditions of increased stringency can be obtained, for example, by increasing the time of soaking or changing the pH of the soak solution, etc.

Competition

Referring to figure 2(ii) antigen ag can be bound to a solid support s and bound to saturation by the original binding molecule c. If a population of mutant phage antibody (or a set of unrelated phage antibody) p is offered to the complex, only those that have higher affinity for antigen ag than c will bind. In most examples, only a minority of population c will be displaced by individuals from population p. If c is a traditional antibody molecule, all bound material can be recovered and bound p recovered by infecting suitable bacteria and/or by use of standard techniques such as PCR.

An advantageous application is where ag is used as a receptor and c the corresponding ligand. The recovered bound population p is then related structurally to the receptor binding site/and or ligand. This type of specificity is known to be very useful in the pharmaceutical

industry.

Another advantageous application is where ag is an antibody and c its antigen. The recovered bound population p is then an anti-idiotype antibody which have numerous uses in research and the diagnostic and pharmaceutical industries.

In some instances it may prove advantageous to pre-select population p. For example, in the anti-idiotype example above, p can be absorbed against a related antibody that does not bind the antigen.

However, if c is a phage antibody, then either or both c and p can advantageously be marked in some way to both distinguish and select for bound p over bound c. This marking can be physical, for example, by pre-labelling p with biotin; or more advantageously, genetic. For example, c can be marked with an EcoB restriction site, whilst p can be marked with an EcoK restriction site (see Carter, P. et al., 1985, Nucl. Acids Res. 13, 4431-4443). When bound p+c are eluted from the antigen and used to infect bacteria, there is restriction (and thus no growth) of population c (i.e. EcoB restricting bacteria in this example). Any phage that grew, would be greatly enriched for those individuals from p with higher binding affinities. Alternatively, the genetic marking can be achieved by marking p with new sequences, which can be used to specifically amplify p from the mixture using PCR.

The novel structure of the phage antibody molecule can be used in a number of other applications some examples of which are:

Signal Amplification

Acting as a novel molecular entity in itself, phage antibodies combine the ability to bind the specific antigen with the amplification, if the major coat protein is used to attach another moiety. This moiety can be attached via immunological, chemical, or any other means and can be used,

for example, to label the complex with detection reagents or cytotoxic molecules for use in vivo or in vitro.

Physical Detection

The size of the phage antibody can be used as a marker particularly with respect to physical methods of detection such as electron microscopy and/or some biosensors, eg. surface plasmon resonance.

Diagnostic Assays

The phage antibody molecule also has advantageous uses in diagnostic assays, particularly where separation can be effected using its physical properties for example centrifugation, filtration etc.

In order that the invention is more fully understood, embodiments will now be described in more detail by way of example only and not by way of limitation with reference to the figures described below.

Figure 1 shows the basic structure of the simplest antibody molecule IgG.

Figure 2 shows schematically novel screening techniques which utilise the unique properties of phage antibodies.

Figure 3 shows a scheme for the construction of vectors.

Figure 4 shows the nucleotide sequences for the oligonucleotides and vectors. All sequences are drawn 5' to 3' and are numbered according to Beck et al., 1978, Nucl. Acid Res., 5: 4495-4503. 4.1 shows the sequences of the ologonucleotides used for mutagenesis (oligo's 1 and 2) or sequencing (oligo 3). The sequences shown were synthesized on an Applied Biosystems, olgonucleotide synthesizer and are complementary to the single stranded form of fd-tet (they are in the anti-sense form with respect to gene III). 4.2 shows the sequences of the various constructs around the gene III insertion site. these sequences are drawn in the sense orientation with respect to gene III; a) fd-tet (and

FDTdBst) b) FDTPs/Bs and c) FDTPs/Xh. The key restriction enzyme sites are shown along with the immunoglobulin amino acids contributed by the vectors, (amino acid single letter code is used, see Harlow, E., and Lane, D., 1988 supra.).

Figure 5 shows the nucleotide and amino acid sequences for scFv in the vector scFvD1.3 myc. This gives the sequence of the anti-lysozyme single chain Fv and surrounding sequences in scFvD1.3 myc showing the N-terminal pel B signal peptide sequence and the C-terminal myc tag sequence (Ward, E.S., et al., 1989, supra.). Also highlighted is the peptide sequence linking the V_H and V_L regions. The amino acid sequence is represented above the nucleotide sequence by the single letter code, see Harlow, E., and Lane, D., 1988 supra..

Figure 6 shows the effect of varying the amount of supernatant on the binding of phage antibodies to lysozyme in graphical form. Each point is the average of duplicate samples. Lysozyme was coated at 1 mg/ml in 50 mM $NaHCO_3$.

Figure 7 shows the effect of varying the coating concentration on the binding of phage antibodies to lysozyme in graphical form. Each point is the average of duplicate samples. Lysozyme was coated with the specified concentration of either BSA or lysozyme.

Figure 8 shows the sequence around the cloning site in gene III of fd-Cat2.

Figure 9 shows the binding of pAb (D1.3) to lysozymes. Binding of phage as detected by ELISA to (a) hen egg-white lysozyme (HEL) (b) turkey egg-white lysozyme (TEL), (c) human lysozyme (HUL), (d) bovine serum albumin (BSA). A further control of (c) fd-CAT1 to HEL.

Figure 10 shows a map of fab D1.3 in pUC19.

Figure 11 shows the ELISA results providing a comparison of lysozyme-binding by phage-Fab and phage-ScFv. Vector=fd-CAT2 (example 5); fdSCFV(OX)=pAbNQ11 (Example 9); fdVHCH1(D1.3)=grown in normal cells (ie. no light chain, see

example 7); fdfab-fdfab-fdVHCH1 (D1.3) grown in cells containing D1.3 light chain; fdSCFV (D1.3)=pAbD1.3.

Figure 12 shows oligonucleotide probing of affinity purified phage. 10^{12} phage in the ratio 1 pAb (D1.3) in

5   4×10^4 fd-CAT1 phages were affinity purified and probed with an oligonucleotide specific for pAb (D1.3) (a) filter after one round of affinity purification (900 colonies total) and, (b) after two rounds (372 colonies total).

10   Figure 13 shows the sequence of the anti-oxazalone antibody NQ11.

Figure 14 shows the ELISA results for binding of pAb NQ11 and pAb D1.3 and Vector FDTPs/xh to specified antigens.

15   Figure 15 shows the sequence surrounding phoA insertion in fd-phoA1. The restriction sites used for cloning are shown, as well as the amino acids encoded by phoA around the insertion site. The first five amino acids of the mature fusion come from gene 3.

20   Figure 16(1) shows the structure of gene 3 and figure 16(2) shows the peptide linker sites A and B.

Figure 17 shows schematically the protocol for PCR assembly of mouse VH and VLK repertoires for phage display described in example 16.

25   Figure 18 shows examples of the final products obtained with the procedure of example 16. Lanes a and b

show the products of the initial PCR using heavy and light chain primers respectively; lane c shows the complete assembled 700bp product before final digestion with Notl and Apall; M1, M2 markers ∅174 Hae III digest

5   and 123 base pair ladder (BRL Limited, P.O. Box 35, Washington Road, Paisley, Scotland) respectively.

Figure 19 shows the binding of ^{125}I-PDGF-BB to fd h-PDGFB-R phage in immunoprecipitation assay and comparison to fd TPs/Bs and no phage controls; binding is expressed

10   as a percentage of the total ^{125}I-PDGF-BB added to the incubation·

Figure 20 shows the displacement of ^{125}I-PDGF-BB bound to fd-h-PDGFB-R phage using unlabelled PDGF-BB measured using an immunoprecipitation assay. Binding is

15   expressed as a percentage of the total ^{125}I-PDGF-BB added to the incubation·

Figure 21 shows the displacement of ^{125}I-PDGF-BB bound to fd-h-PDGFB-R phage using unlabelled PDGF-BB measured using an immunoprecipitation assay. Non-

20   specific binding of ^{125}I-PDGF-BB to vector phage fdTPs/Bs in the absence of added unlabelled PDGF was deducted from each point.

Figure 22 shows the results of an ELISA of lysozyme binding by pCAT-3 ScFv D1.3 phagemid in comparison with

25   pCAT-3 vector (both rescued by M13KO7) and fdCAT2 ScFv D1.3 as described in example 17. The ELISA was performed

as described in example 6 with modifications detailed in example 17.

The following procedures used by the present applicants are described in Sambrook, J. et al., 1989, supra.: restriction digestion, ligation, preparation of competent cells (Hanahan method), transformation, analysis of restriction enzyme digestion products on agarose gels, purification of DNA using phenol/chloroform, 5'-end labelling of oligonucleotides, filter screening of bacterial colonies, preparation of 2xTY medium and plates, preparation of tetracycline stock solutions, PAGE of proteins, preparation of phosphate buffered saline.

All enzymes were supplied by New England Biolabs (CP Laboratories, PO Box 22, Bishop's Stortford, Herts., England) and were used according to manufacturer's instructions unless otherwise stated.

The vector fd-tet (Zacher, A.N. et al., 1980, supra) was obtained from the American Type Culture Collection (ATCC

No. 37000) and transformed into competent TG1 cells
(genotype: K12δ (lac-pro), sup E, thi, hsdD5/F'traD36, pro
A+B+, Lac 1^q, lac δM15).

Viral particles were prepared by growing TG1 cells
containing the desired construct in 10 to 100 mls 2xTY
medium with 15 µg/ml tetracycline for 16-24 hours. The
culture supernatant was collected by centrifugation for 10
mins at 10,000 rpm in an 8 x 50 ml rotor, Sorval RC-5B
centrifuge. Phage particles were precipitated by adding
1/5th volume 20% polyethylene glycol (PEG)/2.5M NaCl and
leaving at 4°C for 1 hour. These were spun for 15 minutes
as described above and the pellets resuspended in 10 mM
Tris/HCI pH 8, 1 mM EDTA to 1/100th of the original volume.
Residual bacteria and undissolved material were removed by
spinning for 2 minutes in a microcentrifuge. Single
stranded DNA for mutagenesis or sequencing was prepared from
concentrated phage according to Sambrook, J., et al., 1989,
supra.

Example 1

Design of Insertion Point Linkers and Construction of
Vectors

The vector fd-tet has two BstEII restriction sites
flanking the tetracycline resistance gene (fig 3). Since
the strategy for inserting the V_H fragments was to ligate
them into a newly inserted BstEII site within gene III, it
was advantageous to delete the original BstEII sites from
fd-tet. This was achieved by digesting fd-tet with the
restriction enzyme BstEII, filling-in the 5' overhangs and
re-ligating to generate the vector FDTδBst. Digestion of
fd-tet with BstEII (0.5 units/µl) was carried out in 1x KGB
buffer (100 mM potassium glutamate, 25 mM Tris-acetate (PH
7.5), 10 mM magnesium acetate, 50 µg/ml bovine serum
albumin, 0.5 mM (Sambrook, J., et al., 1989, supra.) with
DNA at a concentration of 25 ng/µl. The 5' overhang was
filled in, using 2x KGB buffer, 250 µM each dNTP's

(Pharmacia Ltd., Pharmacia House, Midsummer Boulevard, Milton Keynes, Bucks., UK.) and Klenow Fragment (Amersham International, Lincoln Place, Green End, Aylesbury, Bucks., UK) at 0.04 units/ μl. After incubating for 1 hour at room temperature, DNA was extracted with phenol/chloroform and precipitated with ethanol.

Ligations were carried out at a DNA concentration of 50ng/μl for 1 hour at room temperature using T4 DNA ligase (40 units/μl). Ligations were transformed into competent TG1 cells and plated onto TY plates supplemented with 15 μg/ml tetracycline. Colonies were picked into 25 mls of 2xTY medium supplemented with 15 μg/ml tetracycline and grown overnight at 37°C.

Double stranded DNA was purified from the resulting clones using the gene-clean II kit (Bio101 Inc., PO Box 2284, La Jolla, California, 92038-2284, USA.) and according to the small scale rapid plasmid DNA isolation procedure described therein. The orientation of 5 of the resulting clones was checked using the restriction enzyme Cla1. A clone was chosen which gave the same Cla1 pattern as fd-tet, but which had no BstE II sites.

In vitro mutagenesis of FDTδBst was used to generate vectors that facilitated cloning of antibody fragments downstream of the gene III signal peptide and in frame with the gene III coding sequence. The oligonucleotide directed mutagenesis system, version 2 (Amersham International) was used with oligo 1 (figure 4) to create FDTPs/Bs. The sequence of FDTPs/Bs (figure 4) was confirmed using the sequenase version 2.0 kit (USB Corp., PO Box 22400, Cleveland, Ohio, 44122, USA.) with oligo 3 (figure 4) as a primer.

A second vector FDTPs/Xh (to facilitate cloning of single chain F_V fragments) was generated by mutagenising FDTPs/Bs with oligo 2 according to the method of Venkitaraman, A.R., Nucl. Acid Res. 17, p 3314. The

sequence of FDTPs/Xh (figure 4) was confirmed using the sequenase version 2.0 kit (USB Corp.) with oligo 3 as a primer.

Clearly, alternative constructions will be apparent to those skilled in the art. For example, M13 and/or its host bacteria could be modified such that its gene III could be disrupted without the onset of excessive cell death; the modified fd gene III, or other modified protein, could be incorporated into a plasmid containing a single stranded phage replication origin, such as pUC119, superinfection with modified phage such as KO7 would then result in the encapsulation of the phage antibody genome in a coat partially derived from the helper phage and partly from the phage antibody gene III construct.

The detailed construction of a vector such as FDTPs/Bs is only one way of achieving the end of a phage antibody. For example, techniques such as sticky feet cloning/mutagenesis (Clackson, T. and Winter, G. 1989 Nucl. Acids. Res., 17, p 10163-10170) could be used to avoid use of restriction enzyme digests and/or ligation steps.

Example 2.

Insertion of Immunoglobulin F_V Domain into Phage Antibody

The plasmid scFv D1.3 myc (gift from G. Winter and A. Griffiths) contains V_H and V_L sequences from the antibody D1.3 fused via a peptide linker sequence to form a single chain F_V version of antibody D1.3. The sequence of the scF$_V$ and surrounding sequences in scFvD1.3 myc is shown in figure 5.

The D1.3 antibody is directed against hen egg lysozyme (Harper, M. et al., 1987, Molec. Immunol. 24, 97-108) and the scFv form expressed in E. coli has the same specificity (A. Griffiths and G. Winter personal Communication).

Digestion of scFv D1.3 myc with PstI and XhoI, excises a fragment of 693 bp which encodes the bulk of the scFv. Ligation of this fragment into FDTPs/Xh cleaved with PstI

and XhoI gave rise to the construct FDTSCFVD1.3 encoding the gene III signal peptide and first amino acid fused to the complete D1.3 scFv, followed by the mature gene III protein from amino acid 2.

The vector FDTPs/Xh was prepared for ligation by digesting with the PstI and XhoI for 2 hours followed by digestion with calf intestinal alkaline phosphatase (Boehringer Mannheim UK Ltd., Bell Lane, Lewes, East Sussex, BN7 1LG) at one unit/ul for 30 minutes at 37°C. Fresh calf intestinal alkaline phosphatase was added to a final total concentration of 2 units/ul and incubated for a further 30 minutes at 37°C. The reaction was extracted three times with phenol/chloroform, precipitated with ethanol and dissolved in water. The insert from scFvD1.3 myc was excised with the appropriate restriction enzymes, extracted twice with phenol/chloroform, precipitated with ethanol and dissolved in water. Ligations were carried out as described in example 1 except both vector and insert samples were at a final concentration of 5 ng/ul each. The formation of the correct construct was confirmed by sequencing as described in example 1.

To demonstrate that proteins of the expected size were produced, virions were concentrated by PEG precipitation as described above, and the equivalent of 2mls of supernatant was loaded onto an 18% SDS polyacrylamide gel. After electrophoresis, the gel was soaked in gel running buffer (50 mM Tris, 380 mM Glycine, 0.1%SDS) with 20% methanol for 15 minutes. Transfer to nitrocellulose filter was executed in fresh 1x running buffer/20% methanol using TE70 Semi Phor a semi-dry blotting apparatus (Hoeffer, 654 Minnesota Street, Box 77387, San Francisco, California 94107, USA.).

After transfer, the filter was blocked by incubation for 1 hour in a 2% solution of milk powder (Cadbury's Marvel) in phosphate buffered saline (PBS). Detection of F_v and V_H protein sequences in the phage antibody fusion proteins was effected by soaking the filter for 1 hour with a 1/1000 dilution (in 2% milk powder) of a rabbit polyclonal

antiserum raised against affinity purified, bacterially expressed F$_V$ fragment (gift from G. Winter). After washing PBS (3 x 5 minute washes), bound primary antibody was detected using an anti-rabbit antibody conjugated to horseradish peroxidase (Sigma, Fancy Road, Poole, Dorset, BH17 7NH, UK.) for 1 hour. The filter was washed in PBS/0.1% triton X-100 and developed with 0.5 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (DAB), 0.02% cobalt chloride, 0.03% hydrogen peroxide in PBS.

The results show that with FDTVHD1.3 (from example 3) and FDTSCVFVD1.3, a protein of between 69,000 and 92,500 daltons is detected by the anti-F$_V$ serum. This is the expected size for the fusion proteins constructed. This product is not observed in supernatants derived from fd-tet, FDT6Bst or FDTPs/Xh.

Example 3.

Insertion of Immunoglobulin V$_H$ Domain into Phage Antibody

The VH fragment from D1.3 was generated from the plasmid pSW1-VHD1.3-TAG1 (Ward, E.S. et al., 1989 supra.). Digestion with Pst1 and BstEII generates the fragment shown between positions 113 and 432 in figure 5. Cloning of this fragment into the Pst1 and BstEII sites of FDTPs/Bs gave rise to the construct FDTVHD1.3 which encodes a fusion protein with a complete V$_H$ inserted between the first and third amino acids of the mature gene III protein (amino acid two has been deleted).

The methods used were exactly as in example 2 except that the vector used was FDTPs/Bs digested with Pst1 and BstEII.

Example 4.

Analysis of Binding Specificity of Phage Antibodies

The binding of the various phage antibodies to the specific antigen, lysozyme, was analysed using ELISA techniques. Phage antibodies were grown in E.coli and phage antibody particles were precipitated with PEG as in the materials and methods. Bound phage antibody particles were detected using polyclonal rabbit serum raised against the

closely related phage M13.

ELISA plates were prepared by coating 96 well plates (Falcon Microtest III flexible plate. Falcon: Becton Dickinson Labware, 1950 Williams Drive, Oxnard, Califorrna, 93030, USA.) with 200 ul of a solution of lysozyme (1mg/ml unless otherwise stated) in 50 mm $NaHCO_3$ for 16-24 hours. Before use, this solution was removed, the plate rinsed several times in PBS and incubated with 200 ul of 2% milk powder/PBS for 1 hour. After rinsing several times with PBS, 100 ul of the test samples were added and incubated for 1 hour. Plates were washed (3 rinses in 0.05% Tween 20/PBS followed by 3 rinses in PBS alone). Bound phage antibodies were detected by adding 200 ul/well of a 1/1000 dilution of sheep anti-M13 polyclonal antiserum (gift from G. Winter) in 2% milk powder/PBS and incubating for 1 hour. After washing as above, plates were incubated with biotinylated anti-sheep antibody (Amersham International) for 30 minutes. Plates were washed as above, and incubated with streptavidin-horseradish peroxidase complex (Amersham International). After a final wash as above, 0.5 mg/ml ABTS substrate in citrate buffer was added (ABTS=2'2'-azinobis(3-ethylbenzthiazoline sulphonic acid); citrate buffer =50 mM citric acid, 50 mM tri-sodium citrate at a ratio of 54:46). Hydrogen peroxide was added to a final concentration of 0.003% and the plates incubated for 1 hour. The optical density at 405 nm was read in a Titertek multiskan plate reader.

Figure 6 shows the effect of varying the amount of phage antibody. 100 ul of various dilutions of PEG precipitated phage were applied and the amount expressed in terms of the original culture volume from which it was derived. Signals derived from both the scFv containing phage antibody (FDTSCFVD1.3) and the V_H containing phage antibody (FDTVHD1.3) were higher than that derived from the phage antibody vector (FDTPs/Xh). The highest signal to noise ratio occurs using the equivalent of 1.3 mls of culture.

Figure 7 shows the results of coating the plates with varying concentrations of lysozyme or bovine serum albumin (BSA). The equivalent of 1 ml of the original phage antibody culture supernatant was used. The signals from supernatants derived from FDTSCFVD1.3 were again higher than those derived from FDTPs/Xh when lysozyme coated wells were used. There was no significant difference between these two types of supernatant when the plates were coated with BSA. Broadly speaking the level of signal on the plates is proportional to the amount of lysozyme coated. These results demonstrate that the binding detected is specific for lysozyme as the antigen.

Example 5.

Construction of fd Cat 2

It would be useful to design vectors that enable the use of restriction enzymes that cut DNA infrequently, thus avoiding unwanted digestion of the antibody gene inserts within their coding sequence. Enzymes with an eight base recognition sequence are particularly useful in this respect, for example Not1 and Sfi1. Chaudhary et al (PNAS 87 p1066-1070, 1990) have identified a number of restriction sites which occur rarely in antibody variable genes. The applicant has designed and constructed a vector that utilises two of these sites, as an example of how this type of enzyme can be used. Essentially sites for the enzymes ApaL1 and Not1 were engineered into FDTPs/Xh to create fdCAT2.

The oligonucleotide:

5'ACT TTC AAC AGT TTC TGC GGC CGC CCG TTT GAT CTC GAG CTC CTG CAG TTG GAC CTG TGC ACT GTG AGA ATA GAA 3'

was synthesised (supra fig 4 legend) and used to mutagenise FDTPs/Xh using an in vitro mutagenesis kit from Amersham International as described in example 1, to create fd-CAT2. The sequence of fd-CAT2 was checked around the site of manipulation by DNA sequencing. The final sequence around the insertion point within gene III is shown in figure 8.

Example 6

Specific Binding of Phage-antibody(pAb) to Antigen

The binding of pAb D1.3 (FDTSCFVD1.3 of example 2) to lysozyme was further analysed by ELISA.

Methods.

1. Phage growth.

Cultures of phage transduced bacteria were prepared in 10-100 mls 2 x YT medium with 15 μg/ml tetracycline and grown with shaking at 37°C for 16-24 hrs. Phage supernatant was prepared by centrifugation of the culture (10 min at 10,000 rpm, 8 x 50 ml rotor, Sorval RC-5B centrifuge). At this stage, the phage titre was 1 - 5 x 10^{10} ml-^{1} transducing units. The phage were precipitated by adding 1/5 volume 20% polyethylene glycol, 2.5 M NaCl, leaving for 1 hr at 4°C, and centrifuging (supra). The phage pellets were resuspended in 10 mM Tris-HCl, 1mM EDTA pH 8.0 to 1/100th of the original volume, and residual bacteria and aggregated phage removed by centrifugation for 2 min in a bench microcentrifuge.

ELISA

Plates were coated with antigen (1 mg ml-^{1} antigen) and blocked as described in example 4. 2 x 10^{10} phage transducing units were added to the antigen coated plates in phosphate buffered saline (PBS) containing 2% skimmed milk powder (MPBS). Plates were washed between each step with three rinses of 0.5% Tween-20 in PBS followed by three rinses of PBS. Bound phage was developed by incubating with sheep anti-M13 antisera and detected with horseradish peroxidase (HRP) conjugated anti-goat serum (Sigma, Poole, Dorset, UK) and ABTS (2'2'-azinobis (3-ethylbenzthiazoline sulphonic acid). Readings were taken at 405 nm after a suitable period. The results (figure 9) show that the antibody bearing-phage had the same pattern of reactivity as the original D1.3 antibody (Harper, M., Lema, F., Boulot, G., and Poljak, F.J. (1987) Molec. Immunol. 24, 97-108), and bound to hen egg-white lysozyme, but not to turkey egg-white lysozyme, human lysozyme or bovine serum albumin. The specificity of the phage is particularly illustrated by the

lack of binding to the turkey egg-white lysozyme that differs from hen egg-white lysozyme by only 7 amino acids.

Example 7

Expression of Fab D1.3 in fd CAT2

The aim of this example was to demonstrate that the scFv format used in example 2 was only one way of displaying antibody fragments in the pAb system. A more commonly used antibody fragment is the Fab fragment (figure 1) this example describes the construction of a pAb that expresses a Fab-like fragment on its surface and shows that it binds specifically to its antigen. The applicant chose to express the heavy chain of the antibody fragment consisting of the VH1 and CH1 domains in the pAb itself and to co-express the light chain in the same cell; the VH and CH1 regions of anti-lysozyme antibody D1.3 were cloned in fd CAT2, and the corresponding light chain cloned in plasmid pUC19. The light chain associates with the heavy chain-gene III fusion as it does when the heavy and light chains are expressed as independant proteins (Skerra, A and Pluckthun, A. Science 240, p1038-1040 (1988)).

It is possible to express the light chain from within the pAb genome by, for example, cloning an expression cassette into a suitable place in the phage genome. Such a suitable place would be the intergenic region which houses the multicloning sites engineered into derivative of the related phage M13 (see, for example, Yanisch-Perron, C. et al., Gene 33, p103-119, (1985)).

The starting point for this example was the clone Fab D1.3 in pUC19, a map of which is shown in figure 10. The sequence encoding the VH-CH1 region was PCR amplified from Fab D1.3 in pUC19 using oligonucleotides KSJ 6 and 7, which retain the Pst I site at the 5'end and introduce a Xho I site at the 3'end, to facilitate cloning into fd CAT2. The sequences for the oligonucleotides KSJ 6 and 7 are shown below.

KSJ6: 5' AGG TGC AGC TGC AGG AGT CAG G 3'

KSJ7: 5' GGT GAC CTC GAG TGA AGA TTT GGG CTC AAC TTT C 3'

PCR conditions were as described in example II, except that thirty cycles of PCR amplification were performed with denaturation at 92°C for 45 seconds, annealing at 55°C for 1 minute and extension at 72°C for 1 minute. The template used was TG1 cells containing Fab D1.3 in pUC19 resuspended in water and boiled (see example 12). This regime resulted in amplification of the expected fragment of approximately 600bp. This fragment was cut with Pst I and Xho I, purified from an agarose gel and ligated into Pst 1/Xho 1-cut fd CAT2 as described in example 12. Part of the ligation was transformed into E.coli MC1061 (Available from, for example Clontech Laboratories Inc, Palo Alto, California) and colonies identified by hybridisation with the oligonucleotide D1.3CDR3A as described in example 10. The presence of the VHCH1 gene fragment was likewise confirmed by PCR, using oligonucleotides KSJ6 and 7. A representative clone was called fd CAT2: VHCH1 D1.3.

The heavy chain was deleted from fab D1.3 in pUC19 by Sph I cleavage of fab D1.3 plasmid DNA. The 2.7Kb fragment containing pUC19 and the light chain gene was purified from a TAE agarose gel, and 10ng of this DNA self-ligated and transformed into competent E.coli TG1. Cells were plated on 2YT agar containing ampicillin (100μg/ml) and incubated at 30°C overnight. The resulting colonies were used to make miniprep DNA (Sambrook et al. supra), and the absence of the heavy chain gene confirmed by digestion with Sph I and Hind III. A representative clone was called LC D1.3 DHC.

An overnight culture of fd CAT2; VHCH1 D1.3 cells was microcentrifuged at 13,000Xg for 10 minutes and 50μl of the supernatant containing phage particles added to 50μl of an overnight culture of LC D1.3 DHC cells. The cells were incubated at 37°C for 10 minutes and plated on 2YT agar containing ampicillin (100μg/ml) and 15mg/ml tetracycline. Phage were prepared from some of the resulting colonies and assayed for their ability to bind lysozyme as described in example 6.

The results (Figure 11) showed that when the heavy and

light chain Fab derivatives from the original anitbody D1.3 were present, the pAb bound to lysozyme. pAb expressing the fd VHCH1 fragment did not bind to lysozyme unless grown in cells also expressing the light chain. This shows that a functional Fab fragment was produced by an association of the free light chain with the VHCH1 fragment fused to gene III and expressed on the surface of the pAb.

Example 8

Isolation of Specific, Desired Phage from a Mixture of Vector Phage.

The applicant purified pAb (D1.3) (originally called FDTSCFVD1.3 in example 2) from mixtures using antigen affinity columns. pAb (D1.3) was mixed with vector fd phage (see table 1) and approximately 10^{12} phage passed over a column of lysozyme-Sepharose (prepared from cyanogen bromide activated sepharose 4B (Pharmacia, Milton Keynes, Bucks, UK.) according to the manufacturers instructions. TG1 cells were infected with appropriate dilutions of the elutes and colonies derived, were analysed by probing with an oligonucleotide that detects only the pAb (D1.3) (see Table 1 and Fig. 12) A thousand fold enrichment of pAb (D1.3) was seen with a single column pass. By growing the enriched phage and passing it down the column again, enrichments of up to a million fold were seen.

Enrichment was also demonstrated using purely immunological criteria. For example, 10^{12} phage (at a ratio of 1 pAb (D1.3) to 4 x 10^6 FDTPs/Bs was subjected to two rounds of affinity selection, and then 26 colonies picked and grown overnight. The phage was then assayed for lysozyme binding by ELISA (as example 6). Five colonies yielded phage with lysozyme binding activities, see table 1, and these were shown to encode the scFv (D1.3) by PCR screening (example 13, using 30 cycles of 1 minute at 92°C, 1 minute at 60°C, 1 minute at 72°C using CDR3PCR1 and oligo 3 (fig. 4) as primers).

Thus very rare pAbs can be fished out of large populations, by using antigen to select and then screen the phage.

Methods

Affinity Chromatography of pAbs

Approximately 10^{12} phage particles in 1ml MPBS were loaded onto a 1 ml lysozyme-Sepharose affinity column which had been prewashed in MPBS. The column was washed in turn with 10 ml PBS; then 10 ml 50 mM Tris-HCl, 500 mM NaCl pH 7.5; then 10ml 50 mM Tris-HCl, 500 mM NaCl pH 8.5; then 5 mls 50 mM Tris-HCl, 500 mM NaCl pH 9.5 (adjusted with triethylamine) and then eluted with 5 ml 100 mM triethylamine. The eluate was neutralised with 0.5 M sodium phosphate buffer pH 6.8 and the phage plated for analysis. For a second round of affinity chromatography, the first column eluate was plated to about 30,000 colonies per petri dish. After overnight growth, colonies were then scraped into 5 ml 2 x YT medium, and a 20 µl aliquot diluted into 10 ml fresh medium and grown overnight. The phage was PEG precipitated as above, resuspended in 1 ml MPBS and loaded onto the column, washed and eluted as above.

Oligonucleotides synthesised:

CDR3PCR1 5'TGA GGA C(A or T) C(A or T)GC CGT CTA CTA CTG TGC 3'

Oligonucleotide probing

40 pmole oligonucleotide VH1FOR (Ward, E. S., et al (1989) Nature 341, 544-546), specific to pAb (D1.3) was phosphorylated with 100 µCi y-32P ATP, hybridised (1pmole/ml) to nitrocellulose filters at 67°C in 6 x SSC buffer for 30 minutes and allowed to cool to room temperature for 30 mins, washed 3 x 1 min at 60°C in 0.1 x SSC.

Example 9

Construction of pAb Expressing Anti-hapten Activity

Oxazalone is a hapten that is a commonly used for studying

the detail of the immune response. The anti-oxazalone antibody, NQ11 has been described previously (E. Gherardi, R. Pannell, C. Milstein, J. Immunol. Method $\underline{126}$ 61-68). A plasmid containing the VH and VL genes of NQ11 was converted to a ScFv form by inserting the BstEII/SacI fragment of SCFVD1.3 myc (nucleotides 432-499 of Fig 5) between the VH and VL genes to generate pSCFVNQ11, the sequence of which is shown in fig 13. This ScFv was cloned into the Pst1/Xho1 site of FdTPs/Xh (as described earlier) to generate pAb NQ11. (NQ11 has an internal Pst1 site and so it was necessary to do a complete digest of pSCFVNQ11 with Xho1 followed by a partial digest with Pst1)

The specific binding of pAb NQ11 was confirmed using ELISA. ELISA plates were coated at 37°C in 50mM NaHCO3 at a protein concentration of 200 µg/ml. Plates were coated with either hen egg lysozyme (HEL), bovine serum albumin (BSA), or BSA conjugated to oxazalone (OX-BSA) (method of conjugation in Makela O., Kaartinen M., Pelkonen J.L.T., Karjalainen K. (1978) J.Exp.Med.$\underline{148}$ 1644). Preparation of phage, binding to ELISA plates, washing and detection was as described in example 6. Samples were assayed in duplicate and the average absorbance after 10 minutes presented in figure 14.

This result demonstrates that the pAb NQ11 binds the correct antigen. Figure 14 also shows that pAb D1.3 and pAb NQ11 bind only to the antigen against which the original antibodies were raised.

Example 10

Enrichment of pAb D1.3 from Mixtures of Other pAb by Affinity Purification

3×10^{10} phage in 10mls of PBSM at the ratios of pAb D1.3 to pAb NQ11 shown in table 2 were passed over a 1ml lysozyme Sepharose column. Washing, elution and other methods were as described in example 8 unless otherwise stated. Elutes from the columns were used to infect TG1 cells which were

then plated out. Colonies were probed with a probe which distinguishes pAb D1.3 from pAb NQ11. The sequence of this oligonucleotide (D1.3CDR3A) is:-

5'GTA GTC AAG CCT ATA ATC TCT CTC 3'

Table 2 presents the data from this experiment. An enrichment of almost 1000 fold was achieved in one round and an enrichment of over a million fold in two rounds of purification. This parallels the result described in example 8.

Example 11

Insertion of the Extracellular Domain of the Human Receptor for Platelet Derived Growth Factor Isoform BB into fdCAT-2

A gene fragment encoding the extracellular domain of the human receptor for platelet derived growth factor isoform BB (h-PDGFB-R) was isolated by amplification using the polymerase chain reaction, of plasmid RP41 (from the American Type Culture collection, Cat. No. 50735), a cDNA clone encoding amino acids 43 to 925 of the PDGF-B receptor (Gronwald, R.G.K. et al., PNAS 85, p3435-3439 (1988), amino acids 1 to 32 constitute the signal peptide). The oligonucleotide primers were designed to amplify the region of the h-PDGFB-R gene corresponding to amino acids 43 to 531 of the encoded protein. The primers also incorporate a unique ApaL1 site at the 5' end of the fragment and a unique XhoI site at the 3' end, to facilitate cloning into the vector fdCAT-2. The sequence of the primers is:

RPDGF1 5'C ACA GTG CAC GTC CTC AAT GTC TCC AGC ACC TTC 3'
RPDGF2 5'GAT CTC GAG CTT AAA GGG CAA GGA GTG TGG CAC 3'

PCR amplification was performed using high fidelity conditions (Eckert, K.A. and Kunkel, T.A., 1990 Nucl, Acids. Research 18 p3739-3744). The PCR mixture contained; 20mM Tris HCl (pH7.3 at 70°C), 50mM KCl, 4mM magnesium chloride, 0.01% gelatin, 1mM each of dATP, dGTP, dCTP, and dTTP, 500ng/ml RP41 DNA, 1µM each primer and 50 units/ml Taq polymerase (Cetus/Perkin-Elmer, Beaconsfield, Bucks, UK)

Thirty cycles of PCR were performed with denaturation at 92°C for 1 minute, annealing at 50°C for 2 min and extension at 72°C for 3 min. This reaction resulted in amplification of a fragment of ca 1500bp as expected.

fdCAT-2 vector DNA was digested with ApaL1 and XhoI (New England Biolabs) according to manufacturers recommendations, extracted with phenol/chloroform and ethanol precipitated (Sambrook et al., supra). Cloning of amplified RP41 DNA into this vector and identification of the desired clones was performed essentially as in example 12 except that probing was with 32-P labelled RPDGF1 and analytical PCR was performed using RPDGF1 and RPDGF2 as primers.

Example 12

Insertion of the Extracellular Domain of the Human Epidermal Growth Factor Receptor into fd-CAT2

A gene fragment encoding the extracellular domain of the human epidermal growth factor receptor (hEGF-R) was isolated by polymerase chain amplification of plasmid pJ3EGF-R (Clark et al. (1988) J. Cell Physiol. 134, p421-428) which contains the gene for hEGF-R (Ullrich, A. et al., Nature 309, p418-425, (1984). The oligonucleotide primers used were designated to amplify the region of the hEGF-R gene corresponding to amino acid 1 of the mature protein through to amino acid 621. The primers also incorporate an unique ApaL1 site at the 5' end of the fragment and an unique Xho 1 site at the 3' end, to facilitate cloning into the vector fd-CAT2. The sequence of the primers is:

Oligo KSJ4:5' GAT CTC GAG GGA CGG GAT CTT AAG CCC ATT CGT TGG 3'

Oligo KSJ5:5' CAG AGT GCA CTG GAG GAA AAG AAA GTT TGC CA 3'

PCR amplification was carried out using high-fidelity conditions (Eckert, K.A. and Kunkel, T.A. 1990 Nucl. Acids Res. 18, 3739-3744). The PCR mixture contained: 20mM TrisHCl (pH 7.3 at 70°C), 50mM KCl, 4mM $MgCl_2$, 0.01%

gelatin, 1mM each of dATP, dGTP, dCTP and dTTP, 500ng/ml pJ3 plasmid, 0.5μM each primer and 50U/ml Taq polymerase (Cetus/Perkin-Elmer). Thirty cycles of PCR amplification were performed with denaturation at 92°C for 1 minute, annealing at 50°C for 2 minutes and extension at 72°C for 3 minutes. This regime resulted in amplification of a fragment of the expected size (approximately 1800bp).

The PCR mixture was extracted with phenol/chloroform and ethanol precipitated (Sambrook et al. supra.) before digestion with ApaL 1 and Xho 1 (New England BioLabs) according to manufacturers recommendations. The fragment was resolved on a 1% Tris-Acetate-EDTA agarose gel (Sambrook et al. supra.) and purified using Geneclean (BIO 101) (Geneclean, La Jolla, San Diego, California, USA) according to manufacturers reccomendations.

fd-CAT2 vector DNA was digested with ApaL 1 and Xho 1 (New England BioLabs) according to manufacturers reccomendations, extracted with phenol/chloroform and ethanol precipitated (Sambrook et al. supra.).

75ng of ApaL 1/Xho 1-digested vector DNA was ligated to 40ng of PCR-amplified ApaL 1/Xho I-digested hEGF-R fragment in 12μl of ligation buffer (66mM TrisHCl (pH7.6), 5mM MgCl$_2$, 5mM dithiothreitol, 100mg/ml bovine scrum albumin, 0.5mM ATP, 0.5mM Spermidine) and 400 units T4 DNA ligase (New England BioLabs) for 16 hours at 16°C.

Two μl of the ligation mixture was transformed into 200μl of competent E.coli MC1061 cells, plated on 2YT agar containing 15mg/ml tetracycline and incubated at 30°C for 20 hours.

Colonies containing hEGF-R were identified by probing with 32p-labelled KSJ 4 oligonucleotide (Sambrook et al. supra.) and the presence of an insert in hybridising colonies confirmed by PCR using the conditions described above. In this case the template DNA was prepared from the colonies by picking some colony material into 100μl of

distilled water and boiling for 10 minutes. 1µl of this mixture was used in a 20µl PCR.

Example 13.

Insertion of a Gene Encoding an Enzyme (Alkaline phosphatase) into fd-CAT2

As an example of the expression of a functional enzyme on the bacteriophage surface, the applicants have chosen bacterial alkaline phosphatase an enzyme that normally functions as a dimer (McCracken, S. and Meighen, E., J. Biol. Chem. 255, p2396-2404, (1980)). The oligonucleotides were designed to generate a PCR product with an Apa L1 site at the 5' end of phoA gene and a Not 1 site at its 3' end, thus facilitating cloning into fd-CAT 2 to create a gene III fusion protein. The oligonucleotides synthesised were:

phoA1:5' TAT TCT CAC AGT GCA CAA ACT GTT GAA CGG ACA CCA GAA ATG CCT GTT CTG 3' and,

phoA2:5' ACA TGT ACA TGC GGC CGC TTT CAG CCC CAG AGC GGC TTT C3'

The sequence of the phoA gene is presented in Chang C. N. et al., Gene 44, p121-125 (1986).

The PCR reaction was carried out in 100µl containing 50mM KCl, 2.5mM MgCl$_2$, 0.01% gelatin, 10mM Tris/HCl pH 8.3, 0.25 units/µl of Taq polymerase (Cetus/Perkin Elmer) and 0.5µg/ml template. The template was the pEK plasmid (described by Chaidaroglou et al., Biochemistry 27 p8338-8343, 1988). The PCR was carried out in a Techne (Techne, Duxford, Cambridge, UK) PHC-2 dri-block using thirty cycles of 1 min at 92°C, 2 min at 50°C, 3 min at 72°C.

The resultant product was extracted with phenol:chloroform, precipitated with ethanol, and the pellet dissolved in 35µl water. Digestion with 0.3 units/µl of Apl L1 was carried out in 150µl volume according to manufacturers instructions for two hours at 37°C. After heat inactivation of the enzyme at 65°C, NaCl was added to a final concentration of 150mM and 0.4 units/µl Not1 enzyme

added. After incubation for 2 hours at 37°C, the digest was extracted with phenol:chloroform and precipitated as above, before being dissolved in 30µl of water. The vector fd-CAT2 was sequentially digested with Apa L1 and NotI according to the manufacturers instructions and treated with calf intestinal alkaline phosphatase as described in example 2. The sample was extracted three times with phenol:chloroform, precipitated with ethanol and dissolved in water. The ligations were performed with a final DNA concentration of 1-2ng/µl of both the cut fd-CAT2 and then digested PCR product. The ligations were transformed into competent TG1 cells and plated on 2xTY tet plates. Identification of clones containing the desired insert was by analytical PCR performed using the conditions and primers above on boiled samples of the resulting colonies. The correct clone containing the phoA gene fused in frame to gene III was called fd-phoA1. The sequence at the junction of the cloning region is given in figure 15.

Example 14

Measuring Enzyme Activity of Phage-enzyme

Overnight cultures of TG1 or KS272 (E.coli cells lacking phoA. Strauch K. L., and Beckwith J. PNAS 85 1576-1580, 1988) cells containing either fd-phoA1 or fd-CAT2 were grown at 37°C in 2xTY with 15µg/ml tetracycline. Concentrated, PEG precipitated phage were prepared as described earlier. Enzyme assays (Malamy, M.H. and Horecker B.L., Biochemistry 3, p1893-1897, (1964)) were carried out at 24°C in a final concentration of 1M Tris/HCl pH 8.0, 1mM nitrophenyl phosphate (Sigma), 1mM MgCl2. 100µl of a two times concentrate of this reaction mixture was mixed with 100µl of the test sample in a 96 well plate. Absorbance readings were taken every minute for 30 minutes at a wavelength of 405nm in a Titretek Mk 2 plate reader. Initial reaction rates were calculated from the rate of change of absorbance using a molar absorbance of 17000.

Standard curves (amount of enzyme vs. rate of change of absorbance) were prepared using dilutions of purified bacterial alkaline phosphatase (Sigma type III) in 10mM Tris/HCl pH 8.0, 1mM EDTA. The number of enzyme molecules in the phage samples were estimated from the actual rates of change of absorbance of the phage samples and comparison to this standard curve.

The results in Table 3 show that alkaline phosphatase activity was detected in PEG precipitated material in the sample containing fd-phoA1 but not fd-CAT2. Furthermore, the level of activity was consistent with the expected number of 1-2 dimer molecules of enzyme per phage. The level of enzyme activity detected was not dependant on the host used for growth. In particular, fd-phoA1 grown on phoA minus hosts showed alkaline phosphatase activity.

Therefore, the phage express active alkaline phosphatase enzyme from the phoA-gene III fusion on the phage surface.

Example 15

Insertion of Binding Molecules into Alternative Sites in the Phage

The availability of an alternative site in the phage for the insertion of binding molecules would open up the possibility of expressing more than one antibody fragment in a single pAb. This may be used to generate single or multiple binding specificities. The presence of two distinct binding activities on a single molecule will greatly increase the utility and specificity of this molecule. It may be useful in the binding of viruses with a high mutational rate such as human immunodeficiency virus. In addition, it may be used to bring antigens into close proximity (eg. drug targetting or cell fusion) or it may act as a "molecular clamp" in chemical, immunological or enzymatic processes.

The vector fd-tet and the derivatives described here, have a single BamH1 site in gene 3. This has previously

been used for the expression of peptide fragments on the
surface of filamentous bacteriophage (Smith GP. (1985)
Science 228 p1315-1317 and de la Cruz et al. (1988) J Biol.
Chem. 263 p4318-4322). This provides a potential
alternative site for the insertion of antibody fragments.

DNA fragments encoding SCFv's from D1.3 or NQ11 were
generated by PCR using the primers shown below. These
primers were designed to generate a fragment with BamH1
sites near both the terminii, to enable cloning into the
BamH1 site of gene3 (see figure 16(1)). The
oligonucleotides used, also ensure that the resulting PCR
product lacks Pst1 and Xho1 restriction sites normally used
for manipulating the SCFv's (see figure 16(1)). This will
facilitate subsequent manipulation of a second antibody
fragment in the usual way at the N terminus of gene 3. The
oligonucleotides used were:-

G3Bam1 5'TTT AAT GAG GAT CCA CAG GTG CAG CTG CAA GAG 3'
G3Bam2 5' AAC GAA TGG ATC CCG TTT GAT CTC AAG CTT 3'.

Preparation of vector and PCR insert

The PCR reaction was carried out in an 80 µl reaction as
described in example 13 using 1ng/µl of template and
0.25U/µl of Taq polymerase and a cycle regime of 94°C for 1
minute, 60°C for 1 minute and 70°C for 2 minutes over 30
cycles. The template was either pSCFvNQ11 (example 9) or
SCFvD1.3 myc (example 2). Reaction products were extracted
with phenol:chloroform, precipitated, dissolved in water and
digested with BamH1 according to manufacturers instructions.
The digest was re-extracted with phenol:chloroform,
precipitated and dissolved in water.

The vector FDTPs/Xh was cleaved with BamH1 and treated
with calf intestinal phosphatase and purified as described
in example 2. Ligations were set up at a vector
concentration of approximately 6ng/µl and a PCR insert
concentration of approximately 3ng/µl. These were ligated
for 2.5 hours at room temperature before transforming into

competent TG1 cells and plating on TYE tet plates. The resultant colonies were probed as described in example 8. DNA was prepared from a number of colonies and the correct orientation and insert size confirmed by restriction digestion with Hind III in isolation or in combination with BamH1. (One Hind III site is contributed by one of the primers and the other by the vector).

Two clones containing a D1.3 insert (FDTBam1 and FDTBam2) and one containing an NQ11 insert (NQ11Bam1) were grown up and phage prepared as described earlier. ELISAs were carried out as described in example 6. No specific signal was found for any of these clones suggesting that the natural BamH1 site is not a suitable site for insertion of a functional antibody (results not shown).

It may be possible to clone into alternative sites to retain binding activity. The peptide repeats present in gene3 may provide such a site (figure 16 blocks A and B). This can be done by inserting a BamH1 site and using the PCR product described above. To facilitate this, the natural BamH1 site was removed by mutagenesis with the oligonucleotide shown below (using an in vitro mutagenesis kit (Amersham International)):-
G3mutδBam 5' CA AAC GAA TGG GTC CTC CTC ATT A 3'
The underlined residue replaces an A residue thereby removing the BamH1 site. DNA was prepared from a number of clones and several mutants lacking BamH1 sites identified by restriction digestion.

The oligonucleotide G3 Bamlink was designed to introduce a BamH1 site at a number of possible sites within the peptide linker sites A and B, see figure 16(2). The sequence of the linker is:
5'CC (G or A) CC ACC CTC GGA TCC (G or A) CC ACC CTC 3'
Its relationship to the peptide repeats in gene III is shown in figure 16.

Example 16

PCR Assembly of Mouse VH and VL kappa (VLK) Repertoires
for Phage Display

5

The principle is illustrated in figure 17. Details
are provided in sections A to F below but the broad
outline is first discussed.

1.   cDNA is prepared from spleen RNA from an appropriate
10   mouse and the VH and VLK repertoires individually
          amplified.   Separately,   primers   reverse   and
          complementary to VH1FOR-2 (domain 1) and VLK2BACK
          (domain 2) are used to amplify an existing scFv-
          containing DNA by PCR.   (The term FOR refers to eg.
15   a primer for amplification of sequences on the sense
          strand resulting in antisense coding sequences. The
          term BACK refers to eg. a primer for amplification
          of sequences on the antisense strand resulting in
          sense coding sequences).   This generates a 'linker'
20.   molecule encoding the linker with the amino acid
          sequence (1 letter code) (GGGGS)$_3$ which overlaps the
          two primary (VH and VLK) PCR products.

2.   The separate amplified VH, VLK and linker sequences
          now have to be assembled into a continuous DNA
25   molecule by use of an 'assembly' PCR.   In the
          secondary 'assembly' PCR, the VH, VLK and linker

bands are combined and assembled by virtue of the above referred to overlaps, to generate an assembled PCR band with VHs and VLKs randomly spliced in frame for expression as scFVs.

5   The assembly PCR is carried out in two stages. Firstly, 7 rounds of cycling with just the three bands present in the PCR, followed by a further 20 rounds in the presence of the flanking primers VH1BACK (referring to domain 1 of VH) and VLKFOR.

10   The nucleotide sequences for these oligonucleotide primers are provided under the section entitled 'Primer Sequences' below. This two stage process, avoids the potential problem of preferential amplification of the first combinations to be

15   assembled.

3.   For cloning into the phage system, the assembled repertoires must be 'tagged' with the appropriate restriction sites. In the example provided below this is illustrated by providing an ApaL1

20   restriction site at the VH end of the continuous DNA molecule and a Not 1 site at the VLK end of the molecule. This is carried out by a third stage PCR using tagged primers. The nucleotide sequences for these oligonucleotide primers are also provided

25   under the section entitled 'Primer Sequences' below. There are however, 4 possible kappa light chain

sequences (whereas there is only a single heavy chain sequence). Therefore 4 oligonucleotide primer sequences are provided for VLK.

5   For this third stage PCR, sets of primers which have 4 and 10 nucleotides after the restriction sites have been used. However, long tags may give better cutting, in which case 15-20 nucleotide overhangs could be used.

Scrupulously clean procedures must be used at all 10 times to avoid contamination during PCR. Negative controls containing no DNA must always be included to monitor for contamination. Gel boxes must be depurinated. A dedicated Geneclean kit (B10 101, Geneclean, La Jolla, San Diego, California, USA) can be 15 used according to manufacturers instructions to extract DNA from an agarose gel. The beads, NaI and the NEW wash should be aliquoted.

All enzymes were obtained from CP Laboratories, P.O. Box 22, Bishop's Stortford, Herts CM20 3DH and the 20 manufacturers recommended and supplied buffers were used unless otherwise stated.

A.   RNA Preparation

RNA can be prepared using many procedures well known 25 to those skilled in the art. As an example, the following protocol (Triton X-100 lysis, phenol/SDS RNase

inactivation) gives excellent results with spleen and hybridoma cells (the addition of VRC (veronal ribosyl complex) as an RNase inhibitor is necessary for spleen cells). Guanidinium isothiocyanate/CsCl procedures (yielding total cellular RNA)also give good results but are more time-consuming.

1. Wash 1 - 5 x 10^7 freshly harvested cells in 50 ml PBS at 800g for 10 minutes.

2. On ice, add 1 ml ice-cold lysis buffer (see later for details) to the pellet and resuspend it with a 1 ml Gilson pipette. Leave on ice for 5 minutes.

3. Spin for 5 minutes at 4°C in a microfuge at 13000 rpm, in precooled tubes.

4. Transfer 0.5 ml of the supernatant to each of two eppendorf tubes containing 60μl 10% (w/v) SDS and 250μl phenol (equilibrated with 100MM Tris-HCl pH 8.0). Vortex hard for 2 minutes, then microfuge (13000rpm) for five minutes at room temperature.

5. Re-extract the aqueous upper phage five times with 0.5 ml of phenol.

6. Precipitate with 1/10 volume 3M sodium acetate and 2.5 volumes ethanol at 20°C overnight or dry ice-isopropanol for 30 minutes.

7. Wash the RNA pellet and resuspend in 50μl water. Use 2.5 to check concentration by measuring the optical density at 260nm and check 2μg on a 1% agarose gel. 40μg

to 107µg of RNA was obtained from spleen cells derived from mice.

Lysis buffer is [10mM Tris-HCl pH 7.4, 1mM MgCl$_2$, 150mM NaCl, 10mM VRC (New England Biolabs), 0.5% (w/v) Triton X-100], prepared fresh.

B.   cDNA Preparation

cDNA can be prepared using many procedures well know to those skilled in the art.   As an example, the following protocol can be used:

1.   Set up the following reverse transcription mix:

	µl
H$_2$O (DEPC-treated)	20
5mM dNTP	10
10 x first strand buffer	10
0.1M DTT	10
FOR primer(s) (10 pmol/µl)	2 (each) (see below)
RNasin (Promega; 40 U/µl)	4

NB

i)   DEPC is diethylpyrocarbonate, the function of which is to inactivate any enzymes that could degrade DNA or RNA

ii) dNTP is deoxynucleotide triphosphate

iii) DTT is dithiothreitol the function of which is as an

antioxidant to create the anaerobic environment necessary for enzyme function.

iv) RNasin is a ribonuclease inhibitor obtained from Promega Corporation, 2800 Woods Hollow Road, Madison, Wisconsin, USA.

2. Dilute 10 µg RNA to 40 µl final volume with DEPC-treated water. Heat at 65°C for 3 minutes and hold on ice for one minute (to remove secondary structure).

3. Add to the RNA the reverse transcription mix (58 µl) and 4 µl of the cloned reverse transcriptase 'Super RT' (Anglian Biotech Ltd., Whitehall House, Whitehall Road, Colchester, Essex) and incubate at 42°C for one hour.

4. Boil the reaction mix for three minutes, cool on ice for one minute and then spin in a microfuge to pellet debris. Transfer the supernatant to a new tube.

10 x first strand buffer is [1.4M KCl, 0.5M Tris-HCl pH 8.1 at 42°C 80mM $MgCl_2$].

The primers anneal to the 3' end. Examples of kappa light chain primers are MJK1FONX, MJK2FONX, MJK4FONX and MJK5FONX (provided under 'Primer Sequences' below) and examples of heavy chain primers are CTG GAC AGG GAT CCA

GAG TTC CA and CTG GAC AGG GCT CCA TAG TTC CA. The two
heavy chain primers are provided as alternatives, the
four light chain primers are provided for kappa light
chains 1-4. Primers annealing to CH1, VLK and VL domains

5   could also be used.

C.   Primary PCRs

      For each PCR and negative control, the following
reactions are set up. In the following, the Vent DNA

10   polymerase sold by C.P. Laboratories Ltd. (New England
Biolabs) address given above. The buffers are as
provided by C.P. Laboratories.

	µl
H$_2$O	32.5
10 x Vent buffer	5
20 x Vent BSA	2.5
5mM dNTPs	1.5
FOR primer 10 pMol/µl)	2.5
BACK primer 10pmol/µl)	2.5

15

20

The FOR and BACK primers are given in the section below
entitled 'Primer Sequences'. For VH, the FOR primer is
VH1FOR-2 and the BACK primer is VH1BACK. For VLK the FOR
primers are MJK1FONX, MJK2FONX, MJK4FONX and MJK5FONX

25   (for the four respective kappa light chains) and the BACK
primer is VK2BACK. Only one kappa light chain BACK

primer is necessary, because binding is to a nucleotide sequence common to the four kappa light chains.

UV this mix 5 minutes. Add 2.5 µl cDNA preparation (from B above), 2 drops paraffin oil (Sigma Chemicals,

5 Poole, Dorset, UK). Place on a cycling heating block, e.g. PHC-2 manufactured by Techne Ltd. Duxford UK, pre-set at 94°C. Add 1µl Vent DNA polymerase under the paraffin. Amplify using 25 cycles of 94°C 1 min, 72°C 2 min. Post-treat at 60°C for 5 min.

10 Purify on a 2% lmp (low melting point agarose/TAE (tri-acetate EDTA) gel and extract the DNA to 20 µl H_2O per original PCR using a Geneclean kit (see earlier) in accordance with the manufacturers instructions.

15 D. Preparation of linker

Set up in bulk (eg. 10 times):

	µl
H_2O	34.3
10 x Vent Buffer	5
20 x Vent BSA	2.5
5mM dNTPs	2
LINKFOR primer (10 pmol/µl)	2.5
LINKBACK primer (10 pmol/µl)	2.5
DNA from fcFv D1.3 (example 2)	1
Vent enzyme	0.2

The FOR and BACK primers are given in the section below entitled 'Primer Sequences'. The FOR primer is LINKFOR and the BACK primer is LINKBACK.

5   Cover with paraffin and place on the cycling heating block (see above) at 94°C. Amplify using 25 cycles of 94°C 1 min, 65°C 1 min, 72°C 2 min. Post-treat at 60°C for 5 min.

Purify on 2% lmp/TAE gel (using a loading dye

10 without bromophenol blue as a 93bp fragment is desired) and elute with SPIN-X column (Costar Limited, 205 Broadway, Cambridge, Ma. USA.,) and precipitation.
Take up in 5µl H_2O per PCR reaction.

15   E.   Assembly PCRs

A quarter of each PCR reaction product (5µl) is used for each assembly. The total volume is 25µl.

For each of the four VLK primers, the following are set up:

20

H_2O	4.95
10 x Vent buffer	2.5
20 X Vent BSA	1.25
5mM dNTPs	0.8

25 UV irradiate this mix for 5 min. Add 5µl each of Vh and Vk band and 1.5µl of linker as isolated from the

preparative gels and extracted using the Geneclean kit as described in C and D above. Cover with paraffin. Place on the cycling heating block preset at 94°C. Add 1µl Vent under the paraffin. Amplify using 7 cycles of 94°C

5   2 min, 72°C 4 min. Then return the temperature to 94°C.

Add 1.5µl each of VH1BACK and the appropriate VKFOR primers MJK1FONX, MJK2FONX, MJK4FONX or MJK5FONX (10 pmol/µl) at 94°C. The primers should have been UV-

10   treated as above. Amplify using 20 cycles of 94°C 1.5 min, 72°C 2.5 min. Post-treat at 60°C for 5 min. Purify on 2% lmp/TAE gel and extract the DNA to 20µl H_2O per assembly PCR using a Geneclean kit (see earlier) in accordance with the manufacturers instructions.

15

F.   Adding Restriction Sites

For each assembly and control set up:

	µl
20 H_2O	36.5
10 x Taq buffer	5
5mM dNTPs	2
FOR primer (10 pmol/µl)	2.5
BACK primer (10 pmol/µl)	2.5
25 Assembly product	1

The FOR and BACK primers are given in the section below entitled 'Primer Sequences'. The FOR primer is any of JK1NOT10, JK2NOT10, JK4NOT10 or JK5NOT10 (for the four respective kappa light chains) for putting a Not1 restriction site at the VLK end. The BACK primer is HBKAPA10 for putting an ApaL1 restriction site at the VH end.

Cover with paraffin and place on the cycling heating block preset at 94°C. Add 0.5 μl Cetus Taq DNA polymerase (Cetus/perkin-Elmer, Beaconsfield, Bucks, UK) under the paraffin. Amplification is carried out using 11 to 15 rounds of cycling (depends on efficiency) at 94°C 1 min, 55°C 1 min, 72°C 2 min. Post-treat at 60°C for 5 min.

10 x Taq buffer is [0.1M Tris-HCl pH 8.3 at 25°C, 0.5M KCl, 15mM MgCl$_2$, 1mg/ml gelatine].

G. Work-up

Purify once with CHCl$_3$/IAA (isoamylalcohol), once with phenol, once with CHCl$_3$/IAA and back-extract everything to ensure minimal losses. Precipitate and wash twice in 70% EtOH. Dissolve in 70μl H$_2$O.

Digest overnight at 37°C with NotI: μl

DNA (joined seq)   70

NEB NotI buffer x 10   10

NEB BSA x 10	10
Notl (10 U/µl)	10

The DNA (joined sequence) above refers to the assembled DNA sequence comprising in the 5' to 3' direction

5   ApaL1 restriction site

VH sequence

Linker sequence

VLK sequence

Not 1 restriction site.

10   The VLK sequence may be any one of four possible kappa chain sequences.

The enzymes Not 1 above, ApaL1 below and the buffers NEB Not 1, NEB BSA above and the NEB buffer 4 (below) are obtainable from CP Laboratories, New England Biolabs

15   mentioned above.

Re-precipitate, take up in 80µl H_2O. Add to this 10µl NEB buffer 4 and 10µl Apal 1.

Add the enzyme ApaL1 in aliquots throughout the day, as it has a short half-life at 37°C.

20   Purify on 2% lmp/TAE gel and extract the DNA using a Geneclean kit, in accordance with the manufacturers instructions. Redigest if desired.

F.   Final DNA product

25   The final DNA product is an approximate 700 bp fragment with Apa L1 and Notl compatible ends consisting

of randomly associated heavy and light chain sequences linked by a linker. A typical molecule of this type is the scFvD1.3 molecule incorporated into fdscFvD1.3 described in example 3. These molecules can then be

5   ligated into suitable fd derived vectors, e.g. fdCAT2 or fd CAT3 (example 1), using standard techniques.

Primer sequences.

10   Primary PCR oligos (restriction sites underlined):

VH1FOR-2   TGA GGA GAC GGT GAC CGT GGT CCC TTG GCC CC

VH1BACK   AGG TSM ARC TGC AGS AGT CWG G

MJK1FONX   CCG TTT GAT TTC CAG CTT GGT GCC

MJK2FONX   CCG TTT TAT TTC CAG CTT GGT CCC

15   MJK4FONX   CCG TTT TAT TTC CAA CTT TGT CCC

MJK5FONX   CCG TTT CAG CTC CAG CTT GGT CCC

VK2BACK   GAC ATT GAG CTC ACC CAG TCT CCA

PCR oligos to make linker:

20   LINKFOR   TGG AGA CTC GGT GAG CTC AAT GTC

LINKBACK   GGG ACC ACG GTC ACC GTC TCC TCA

For adding restriction sites:

HBKAPA10   CAT GAC CAC AGT GCA CAG GTS MAR CTG CAG SAG TCW

25   GG

JK1NOT10   GAG TCA TTC TGC GGC CGC CCG TTT GAT TTC CAG CTT

```
                  GGT GCC

JK2NOT10   GAG TCA TTC TGC GGC CGC CCG TTT TAT TTC CAG CTT

                  GGT CCC

JK4NOT10   GAG TCA TTC TGC GGC CGC CCG TTT TAT TTC CAA CTT

                  TGT CCC

JK5NOT10   GAG TCA TTC TGC GGC CGC CCG TTT CAG CTC CAG CTT

                  GGT CCC
```

Example 17

Insertion of the Extralcellular Domain of the Human
Receptor for Platelet Derived Growth FActor PDGF isoform
BB into fdCAT2

This example is substantially equivalent to example
11. However, in example 11, amino acids 33-42 of the
mature protein were not provided. In this example, the
primer RPDGF3 also includes bases encoding these amino
acids.

A gene fragment encoding the extracellular domain of
the human receptor for platelet derived growth factor
isoform BB (h-PDGFB-R) was isolated by amplification,
using the polymerase chain reaction, of plasmid RP41
(from the American Type Culture collection, Cat. No.
50735), a cDNA clone encoding amino-acids 43 to 925 of
the PDGF-B receptor (Gronwald, R.G.K. et al PNAS 85
p3435-3439 (1988), amino acids 1 to 32 constitute the
signal peptide). The oligonucleotide primers were

designed to amplify the region of the h-PDGFB-R gene corresponding to amino acids 43 to 531 of the encoded protein. The primer RPDGF3 for the N-terminal region also included bases encoding amino acids 33 to 42 of the

5   h-PDGFB-R protein (corresponding to the first ten amino acids from the N-terminus of the mature protein) to enable expression of the complete extracellular domain. The primers also incorporate a unique ApaLl site at the N-terminal end of the fragment and a unique XhoI site at

10   the C terminal end to facilitate cloning into the vector fdCAT2. The sequence of the primers is:

RPDGF3 5' CAC AGT GCA CTG GTC GTC ACA CCC CCG GGG CCA GAG
               CTT GTC CTC AAT GTC TCC AGC ACC TTC GTT CTG 3'

15

RPDGF2 5' GAT CTC GAG CTT AAA GGG CAA GGA GTG TGG CAC 3'

PCR amplification was performed using high fidelity conditions (Eckert, K.A. and Kunkel, T.A. 1990 Nucl Acids

20   Research 18 3739-3744). The PCR mixture contained: 20mM TrisHCl (pH7.3 at 70°C, 50mM KCl, 4mM magnesium chloride, 0.01% gelatin, 1mM each of dATP, dCTP, dGTP and dTTP, 500ng/ml RP41 DNA, 1μM each primer and 50units/ml Taq polymerase (Cetus/Perkin Elmer, Beaconsfield, Bucks,

25   U.K.). Thirty cycles of PCR were performed with denaturation at 92°C for 1 min, annealing at 60°C for 1

min and extension at 72°C for 1.5 min. This reaction
resulted in amplification of a fragment of ca. 1500bp as
expected.

    fdCAT2 vector DNA (see example 5) was digested with
5 ApaL1 and Xhol (New England Biolabs) according to
manufacturers recommendations, extracted with
phenol/chloroform and ethanol precipitated (Sambrook et
al, supra). Cloning of amplified RP41 DNA into this
vector and identification of the desired clones was
10 performed essentially as in example 12 except that
probing was with 32-P labelled RPDGF2 and analytical PCR
was performed using RPDGF3 and RPDGF2.

Example 18

Binding of ^{125}1-PDGF-BB to the Extracellular Domain of
the Human Receptor for Platelet Derived Growth Factor
Isoform BB Displayed on the Surface of fd Phage.
5 Mcasured using an Immunoprecipitation Assay.

    Phage particles, expressing the extracellular domain
of the human platelet derived growth factor isoform BB
receptor (fd h-PDGFB-R), were prepared by growing E.coli
MC1061 cells transformed with fd h-PDGFB-R in 50ml of
10 2xTY medium with 15ug/ml tetracyclin for 16 to 20 hours.
Phage particles were concentrated using polyethylene
glycol as described in example 6 and resuspended in PDGF
binding buffer (25mM HEPES, pH7.4, 0.15mM NaCl, 1mM

magnesium chloride, 0.25% BSA) to 1/33rd of the original volume. Residual bacteria and undissolved material were removed by spinning for 2 min in a microcentrifuge. Immunoblots using an antiserum raised against gene III

5 (Prof. I. Rashed, Konstanz., Germany) show the presence in such phage preparations of a geneIII-h-PDGFB-R protein of molecular mass 125000 corresponding to a fusion between h-PDGFB-R external domain (55000 daltons) and geneIII (apparent molecular mass 70000 on SDS-

10 polyacrylamide gel).

Duplicate samples of 350µl concentrated phage were incubated with ^{125}I-PDGF-BB (78.7fmol, 70nCi, 882Ci/mmol; Amersham International plc, Amersham, Bucks) for 1 hour at 37°C. Controls were included in which fdTPs/Bs vector

15 phage (figure 4(26)) or no phage replaced fd h-PDGFB-R phage. After this incubation, 10ul of sheep anti-M13 polyclonal antiserum (a gift from M. Hobart) was added and incubation continued for 30 min at 20°C. To each sample, 40ul (20ul packed volume) of protein G Sepharose

20 Fast Flow (Pharmacia, Milton Keynes) equilibrated in PDGF binding buffer was added. Incubation was continued for 30 min at 20°C with mixing by end over end inversion on a rotating mixer. The affinity matrix was spun down in a microcentrifuge for 2 min and the supernatant removed by

25 aspiration. Non-specifically bound $_{125}$I-PDGF-BB was removed by resuspension of the pellet in 0.5ml PDGF

binding buffer, mixing by rotation for 5 min, centrifugation and aspiration of the supernatant, followed by two further washes with 0.5ml 0.1% BSA, 0.2% Triton-X-100. The pellet finally obtained was

5   resuspended in 100ul PDGF binding buffer and counted in a Packard gamma counter. For displacement studies, unlabelled PDGF-BB (Amersham International) was added to the stated concentration for the incubation of $1_{25}1$-PDGF-BB with phage.

10   $1_{25}1$-PDGF-BB bound to the fd h-PDGFB-R phage and was immunoprecipitated in this assay. Specific binding to receptor phage was 3.5 to 4 times higher than the non-specific binding with vector phage fdTPs/Bs or no phage (fig 19). This binding of ^{125}I-PDGF-BB could be

15   displaced by the inclusion of unlabelled PDGF-BB in the incubation with phage at 37°C (fig 20). At 50nM, unlabelled PDGF-BB the binding of ^{125}I-PDGF-BB was reduced to the same level as the fdTPs/Bs and no phage control. Figure 21 shows the same data, but with the

20   non-specific binding to vector deducted.

These results indicate that a specific saturable site for ^{125}I-PDGF-BB is expressed on fd phage containing cloned h-PDGFB-R DNA. Thus, the phage can display the functional extracellular domain of a cell surface

25   receptor.

Example 19, Construction of Binding molecule-Gene III
Phagemid

It would be useful to improve the transfection
efficiency of the phage-binding molecule system and also
to have the possibility of displaying different numbers
and specificities of binding molecules on the surface of
the same bacteriophage. The applicants have devised a
method that achieves both aims.

The approach is derived from the phagemid system
based on pUC119 [Vieira, J and Messing, J. (1987) Methods
Enzymol. 153:3]. In brief, gene III from fd-CAT2
(example 5) and fd-CAT2 ScFv D1.3 (example 2) was cloned
downstream of the lac promoter in pUC119 in order that
the mutated gene III could be 'rescued' by M13KO7 helper
phage [Vieira, J and Messing, J. et supra.]. The
majority of rescued phage would be expected to contain a
genome derived from the pUC119 plasmid that contains the
binding molecule-gene III fusion and should express
varying numbers of the binding molecule on the surface up
to the normal maximum of 3-5 molecules of gene III on the
surface of wild type phage. The system has been
exemplified below using an antibody as the binding
molecule.

An fdCAT2 containing the single chain Fv form of the
D1.3 antilysozyme antibody was formed by digesting
FDTSCFVD1.3 (example 2) with Pst1 and Xho1, purifying the

fragment containing the scFv fragment and ligating this into Pst1 and Xhol digested fdCAT2. The appropriate clone, called fdCAT2 scFvD1.3 was selected after plating onto 2xTY tetracyclin (15µg/ml) and confirmed by

5   restriction enzyme and sequence analysis.

Gene III from fd-CAT2 (example 5) and fd-CAT2 ScFv D1.3 was PCR-amplified using the primers shown below:

Primer A: TGC GAA GCT TTG GAG CCT TTT TTT TTG GAG ATT TTC AAC G

10   Primer B: CAG TGA ATT CTT ATT AAG ACT CCT TAT TAC GCA GTA TGT TAG C

Primer A anneals to the 5' end of gene III including the ribosome binding site and incorporates a Hind III site. Primer B anneals to the 3' end of gene III at the

15   C-terminus and incorporates two UAA stop codons and an EcoR1 site. One hundred ng of fd-CAT2 and fd-CAT2 ScFv D1.3 DNA was PCR-amplified in a total reaction volume of 50µl as described in example 11, except that 20 cycles of amplification were performed: 94°C 1 minute, 50°C 1

20   minute, 72°C 3 minutes. This resulted in amplification of the expected 1.2Kb fragment from fd-CAT2 and a 1.8Kb fragment from fd-CAT2 ScFv D1.3.

The PCR fragments were digested with EcoR1 and Hind III, gel-purified and ligated into EcoR1- and Hind III-

25   cut and dephosphorylated pUC119 DNA and transformed into E.coli TG1 using standard techniques (Sambrook et al., et

supra). Transformed cells were plated on SOB agar containing 100µg/ml ampicillin and 2% glucose. The resulting clones were called pCAT-3 (derived from fd-CAT2) and pCAT-3 ScFv D1.3 (derived from fd-CAT2 ScFv

5   D1.3).

Example 20, Rescue of Anti-Lysozyme Antibody Specificity from pCAT-3 ScFv D1.3 by M13K07

Single CAT-3 and CAT-3 ScFv D1.3 colonies were

10 picked into 1.5ml 2YT containing 100µg/ml ampicillin and 2% glucose, and grown 6hrs at 30°C. 30µl of these stationary cells were added to 6mls 2YT containing 100µg/ml ampicillin and 2% glucose in 50ml polypropylene tubes (Falcon, Becton Dickinson Labware, 1950 Williams

15 Drive, Oxnard, CA. USA) and grown for 1.5hrs at 30°C at 380rpm in a New Brunswick Orbital Shaker (New Brunswick Scientific Ltd., Edison House 163 Dixons Hill Road, North Mimms. Hatfield, UK). Cells were pelleted by centrifugation at 5,000g for 25 minutes and the tubes

20 drained on tissue paper. The cell pellets were then resuspended in 6mls 2YTamp containing 100µg/ml ampicillin (no glucose) and 4mls 2YT containing 1.25×10^9 p.f.u. ml^{-1} M13K07 bacteriophage added. The mixture was left on ice for 5 minutes followed by growth at 35°C for 45 minutes

25 at 450rpm. A cocktail was then added containing 4µl 100µg/ml ampicillin, 0.5µl 0.1M IPTG and 50µl 10mg/ml

Kanamycin, and the cultures grown overnight at 35°C, 450rpm.

The following day the cultures were centrifuged and phage particles PEG precipitated as described in example
5   6.   Phage pellets were resuspended in 100µl TE (tris-EDTA see example 6) and phage titred on E.coli TG1. Aliquots of infected cells were plated on 2YT containing either 100µg/ml ampicillin to select for pUC119 phage particles, or 50µg/ml Kanamycin to select for the M13 KO7
10 helper phage.   Plates were incubated overnight at 37°C and antibiotic-resistant colonies counted:

DNA	amp^R	Kan^R
pCAT-3	1.8×10^{11} colonies	1.2×10^9 colonies
pCAT-3ScFv D1.3	2.4×10^{11} colonies	2.0×10^9 colonies

15   This shows that the amp^R phagemid particles are infective and present in the rescued phage population at a 100-fold excess over Kan^R M13KO7 helper phage.

Phage were assayed for anti-lysozyme activity by ELISA as described in example 6, with the following
20 modifications:

1)   ELISA plates were blocked for 3 hrs with 2% Marvel/PBS

2)   50µl phage, 400µl 1xPBS and 50µl 20% marvel were mixed end over end for 20 minutes at room temperature
25 before adding 150µl per well.

3)   Phage were left to bind for 2 hours at room

temperature.

4)   All washes post phage binding were:

2 quick rinses PBS/0.5% TWEEN 20

3x2 minute washes -----"-----

5   2 quick rinses PBS no detergent

3x2 minute washes -----"-----

The result of this ELISA is shown in figure 22, which shows that the anibody specificity can indeed be rescued efficiently.

10   It is considered a truism of bacterial genetics that when mutant and wild-type proteins are co-expressed in the same cell, the wild-type protein is used preferentially. This is analagous to the above situation wherein mutant (i.e. antibody fusion) and wild-type gene

15   III proteins (from M13KO7) are competing for assembly as part of the pUC119 phagemid particle. It is therefore envisaged that the majority of the resulting pUC 119 phage particles will have fewer gene III-antibody fusion molecules on their surface than is the case for the

20   purely phage system described for instance in example 2. Such phagemid antibodies are therefore likely to bind antigen with a lower avidity than fd phage antibodies with three or more copies of the antibody fusion on their surfaces (there is no wild-type gene III protein in the

25   system described, for instance, in example 2), and provide a route to production of phage particles with

different numbers of the same binding molecule (and hence

different avidities for the ligand/antigen) or multiple

different binding specificities on their surface, by

using helper phage such as M13KO7 to rescue cells

5 expressing two or more gene III-antibody fusions.

It is also possible to derive helper phage that do

not encode a functional gene III in their genomes (by for

example deleting the gene III sequence or a portion of it

or by incorporating an amber mutation within the gene).

10 These defective phages will only grow on appropriate

cells (for example that provide functional gene III in

trans, or contain an amber suppressor gene), but when

used to rescue phage antibodies, will only incorporate

the gene III antibody fusion encoded by the phagemid into

15 the released phage particle.

Example 21.   Transformation Efficiency of pCAT-3 and

pCAT-3 ScFv D1.3 phagemids

PUC 19, pCAT-3 and pCAT-3 ScFv D1.3 plasmid DNAs,

20 and fdCAT-2 phage DNA was prepared, and used to transform

E.coli TG1, pCAT-3 and pCAT-3 ScFv D1.3 transformations

were plated on SOB agar containing 100µg/ml ampicillin

and 2% glucose, and incubated overnight at 30°C. fdCAT-2

transformations were plated on 2YT agar containing

25 15µg/ml tetracycline and incubated overnight at 37°C.

Transformation efficiences are expressed as colonies per

µg of input DNA.

DNA	Transformation efficiency
pUC 19	1.10^9
pCAT-3	1.10^8
pCAT-3ScFv D1.3	1.10^8
fd CAT-2	8.10^5

As expected, transformation of the phagemid vector is approximately 100-fold more efficient than the parental fdCAT-2 vector. Furthermore, the presence of a ScFv antobdy fragment does not compromise efficiency. This improvement in transformation efficiency is practically useful in the generation of phage antibody libraries that have large repertoires of different binding specificities.

Table 1. Enrichment of pAb (D1.3) from vector population

INPUT RATIO[a]	OUTPUT RATIO		ENRICHMENT[d]
	oligo[b]	ELISA[c]	
pAb : fd-CAT1	pAb : total phage	pAb : total phage	
Single Round			
1 : 4x10^3	43/124		1.3 x 10^3
1 : 4x10^4	2/82		1.0 x 10^3
Two Rounds			
1 : 4x10^4	197/372		2.1 x 10^4
1 : 4x10^5	90/356	3/24	1.0 x 10^5
1 : 4x10^6	27/183	5/26	5.9 x 10^5
1 : 4x10^7	13/278		1.8 x 10^6

Footnotes: [a]Approximately 10^{12} phage with the stated ratio of pAb (D1.3) : FDTPs/Bs were applied to 1 ml lysozyme-sepharose columns, washed and eluted. [b]TG1 cells were infected with the eluted specific binding phage and plated onto TY-tet plates. After incubation overnight at 30-37°C the plates were analysed by hybridisation to the ^{32}P-labelled oligonucleotide VH1FOR (Ward et al op cit) which is specific to pAb D1.3. [c]Single colonies from overnight plates were grown, phage purified, and tested for lysozyme binding. [d]Enrichment was calculated from the oligonucleotide probing data.

Table 2 Enrichment of pAb(D1.3) from mixed pAb population

Input Ratio[1] (pAbD1.3:pAbNQ11)	Output Ratio[2] (pAb D1.3: Total phage)	Enrichment
Single Round		
1 : 2.5 x 10^4	18/460	0.98×10^3
1 : 2.5 x 10^5	3/770	0.97×10^3
1 : 2.5 x 10^6	0/112	-
pAb NQ11 only	0/460	-
Second Round		
1 : 2.5 x 10^4	119/170	1.75×10^4
1 : 2.5 x 10^5	101/130	1.95×10^5
1 : 2.5 x 10^6	102/204	1.26×10^6
1 : 2.5 x 10^7	0/274	-
1 : 2.5 x 10^8	0/209	-
pAb NQ11 only	0/170	-

Notes

1. 10^{10} phage applied to a lysozyme column as in table 1.

2. Plating of cells and probing with oligonucleotide as in table 1, except the oligonucleotide was D1.3CDR3A

Table 3: Enzymic activity of phage-enzyme

Input	ng of enzyme or No. of phage	Rate (OD/hr)	No. of molecules of Enzyme equivalent $(x10^{-11})$
Pure Enzyme	335	34	24.5
Pure Enzyme	177.5	17.4	12.25
Pure Enzyme	88.7	8.7	6.125
Pure Enzyme	44.4	4.12	3.06
Pure Enzyme	22.2	1.8	1.5
Pure Enzyme	11.1	0.86	0.76
No Enzyme	0	0.005	0
fd-phoA1/TG1	1.83×10^{11}	5.82	4.2
fd-CAT2/TG1	1.0×10^{12}	0.155	0.112
fd-phoA1/KS272	7.1×10^{10}	10.32	7.35
fd-CAT2/KS272	8.2×10^{12}	0.038	0.027

FIGURE 1: Antibody structure

Antigen binding site

Fab

Fv

The single domain antibody (dAb, Ward et al. 1989) consists of a single V_H domain.

FIGURE 2: ASSAY FORMATS

2 i) Binding/elution

2 ii) Competion

p - Phage antibody population to be sampled.
ag - Antigen to which binding required.
c - Competitor antibody/ phAb/ligand etc population.
s - Surface (eg plastic, beads etc).
d - Detection system

III (adsorption protein)

VIII (major coat protein)

Cla1

Cla1

FDTET

scale: 1 inch = 1000 bases

Cla1

BstEII

BstEII

Cla1

tetracycline resistance protein (tetA)

fd-tet

cleave with BstEII

fill in with Klenow

re-ligate

FDTδBst

in vitro mutagenesis (oligo1)

FDTPs/Bs

in vitro mutagenesis (oligo 2)

FDTPs/Xh

Figure 3 Scheme for construction of vectors

1.

(1653)
Oligo 1   ACA ACT TTC AAC AGT TGA GGA GAC GGT GAC CGT AAG CTT CTG CAG TTG GAC CTG AGC
GGA GTG AGA ATA (1620)

(1653)
Oligo 2   ACA ACT TTC AAC AGT TTC CCG TTT GAT CTC GAG CTC CTG CAG TTG GAC CTG

(1704)
Oligo 3   GTC GTC TTT CCA GAC GTT AGT

2.

GENE III   GENE III

SIGNAL
CLEAVAGE SITE

(1624)   (1650)
A TCT CAC TCC GCT _____   GAA ACT GTT GAA AGT

```
        Q   V   Q   L   Q              V   T   V   S   S
```
B TCT CAC TCC GCT CAG GTC CAA <u>CTG CAG</u> AAG CTT AC<u>G GTC ACC</u> GTC TCC TCA ACT GTT GAA AGT
                    PstI   BstEII

```
        Q   V   Q   L   Q     L   E   I   K   R
```
C TCT CAC TCC GCT CAG GTC CAA <u>CTG CAG</u> GAG <u>CTC</u> GAG ATC AAA CGG   GAA ACT GTT GAA AGT
                    PstI   XhoI

B= FDTPs/Bs
C= FDTPs/Xh

Figure 4. Sequence of oligos and vectors

Figure 5. Sequence of SCFVD1.5myc   -181-

```
                                     M  K  Y  L  L  P  T  A  L
                 rbs
GCATGCAAATTCTATTTCAAGGAGACAGTCATAATGAAATACCTATTGCCTACGGCAGCC
     10        20        30        40        50        60
SphI
      PelB leader
 A  G  L  L  L  L  A  A  Q  P  A  M  A  Q  V  Q  L  Q  E  S
GCTGGATTGTTATTACTCGCTGCCCAACCAGCGATGGCCCAGGTGCAGCTGCAGGAGTCA
     70        80        90        100       110       120
                                                 PstI


 G  P  G  L  V  A  P  S  Q  S  L  S  I  T  C  T  V  S  G  F
GGACCTGGCCTGGTGGCGCCCTCACAGAGCCTGTCCATCACATGCACCGTCTCAGGGTTC
     130       140       150       160       170       180


 S  L  T  G  Y  G  V  N  W  V  R  Q  P  P  G  K  G  L  E  W
TCATTAACCGGCTATGGTGTAAACTGGGTTCGCCAGCCTCCAGGAAAGGGTCTGGAGTGG
     190       200       210       220       230       240

                      VHD1.3
 L  G  M  I  W  G  D  G  N  T  D  Y  N  S  A  L  K  S  R  L
CTGGGAATGATTTGGGGTGATGGAAACACAGACTATAATTCAGCTCTCAAATCCAGACTG
     250       260       270       280       290       300


 S  I  S  K  D  N  S  K  S  Q  V  F  L  K  M  N  S  L  H  T
AGCATCAGCAAGGACAACTCCAAGAGCCAAGTTTTTCTTAAAAATGAACAGTCTGCACACT
     310       320       330       340       350       360


 D  D  T  A  R  Y  Y  C  A  R  E  R  D  Y  R  L  D  Y  W  G
GATGACACAGCCAGGTACTACTGTGCCAGAGAGAGAGATTATAGGCTTGACTACTGGGGC
     370       380       390       400       410       420

                                        Linker Peptide
 Q  G  T  T  V  T  V  S  S  G  G  G  S  G  G  G  S  G
CAAGGCACCACGGTCACCGTCTCCTCAggtggaggcggttcaggcggaggtggctctggc
     430       440       450       460       470       480
        BstEII


 G  G  G  S  D  I  E  L  T  Q  S  P  A  S  L  S  A  S  V  G
ggtggcggatcggACATCGAGCTCACTCAGTCTCCAGCCTCCCTTTCTGCGTCTGTGGGA
     490       500       510       520       530       540
              SacI


 E  T  V  T  I  T  C  R  A  S  G  N  I  H  N  Y  L  A  W  Y
GAAACTGTCACCATCACATGTCGAGCAAGTGGGAATATTCACAATTATTTAGCATGGTAT
     550       560       570       580       590       600


 Q  Q  K  Q  G  K  S  P  Q  L  L  V  Y  Y  T  T  T  L  A  D
CAGCAGAAACAGGGAAAATCTCCTCAGCTCCTGGTCTATTATACAACAACCTTAGCAGAT
     610       620       630       640       650       660

                   VKD1.3
 G  V  P  S  R  F  S  G  S  G  S  G  T  Q  Y  S  L  K  I  N
GGTGTGCCATCAAGGTTCAGTGGCAGTGGATCAGGAACACAATATTCTCTCAAGATCAAC
     670       680       690       700       710       720


 S  L  Q  P  E  D  F  G  S  Y  Y  C  Q  H  F  W  S  T  P  R
AGCCTGCAACCTGAAGATTTTGGGAGTTATTACTGTCAACATTTTTGGAGTACTCCTCGG
     730       740       750       760       770       780

                                        Myc Tag (TAG1)
 T  F  G  G  G  T  K  L  E  I  K  R  E  Q  K  L  I  S  E  E
ACGTTCGGTGGAGGCACCAAGCTCGAGATCAAACGGGAACAAAAACTCATCTCAGAAGAG
     790       800       810       820       830       840
              XhoI


 D  L  N  *  *
GATCTGAATTAATAATGATCAAACGGTAATAAGGATCCAGCTCGAATTC
     850       860       870       880
                                   EcoRI
```
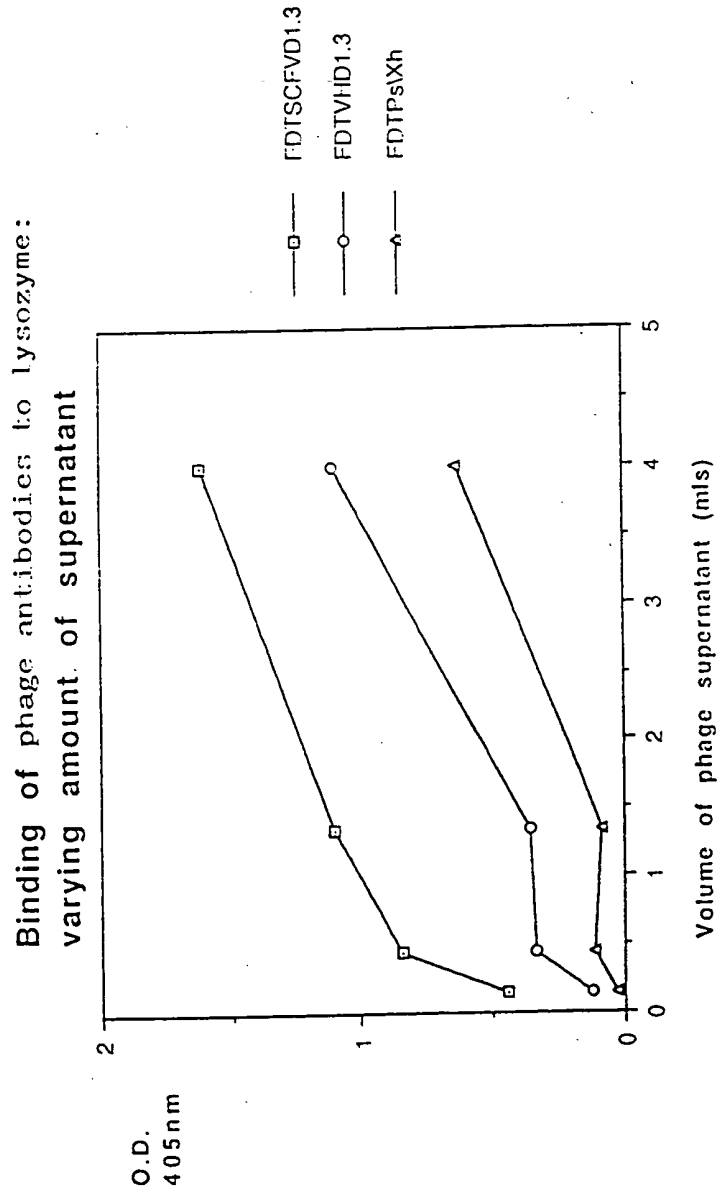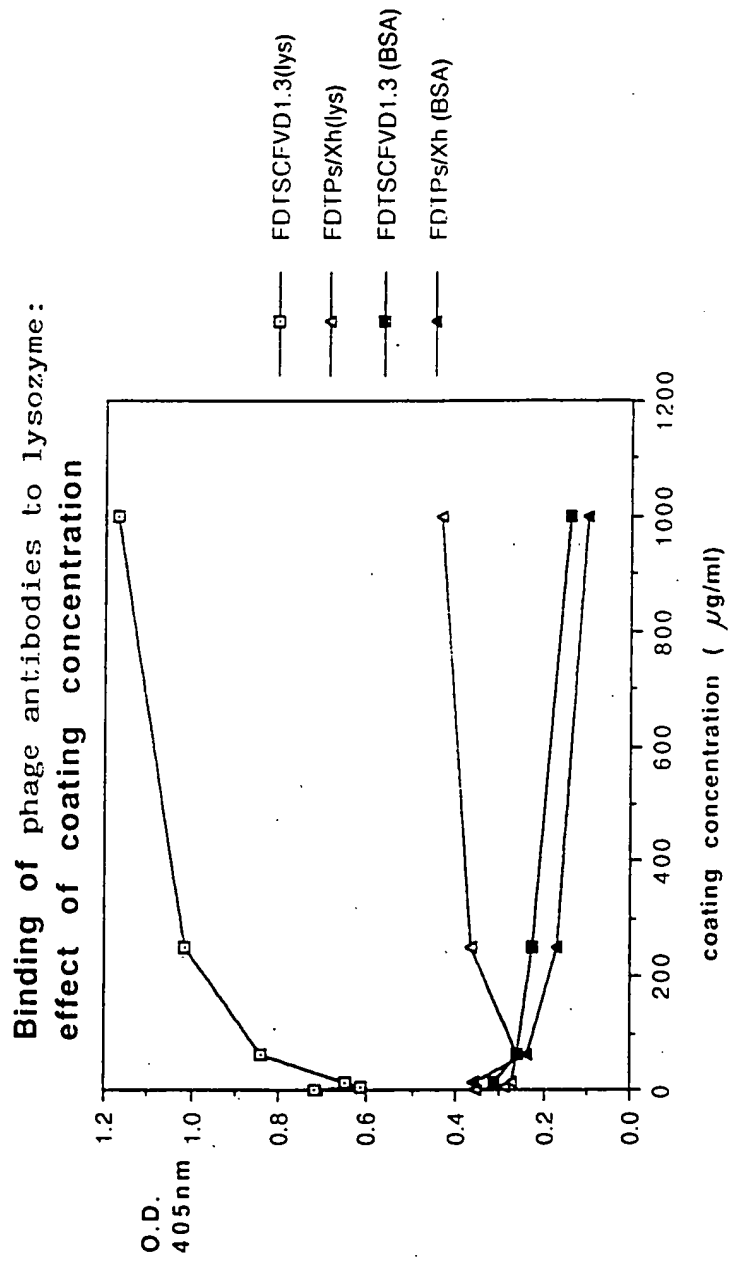
Fig.6.

Binding of phage antibodies to lysozyme: varying amount of supernatant

O.D. 405nm

Volume of phage supernatant (mls)

FDTSCFVD1.3
FDTVHD1.3
FDTPstXh

Methods as described in example 4

Fig. 7

Binding of phage antibodies to lysozyme:
effect of coating concentration

FDTSCFVD1.3(lys)
FDTPs/Xh(lys)
FDTSCFVD1.3 (BSA)
FDTPs/Xh (BSA)

O.D.
405nm

coating concentration (μg/ml)

Methods as described in example 4

Fig. 8. Sequence around the cloning site of
fd-CAT2.
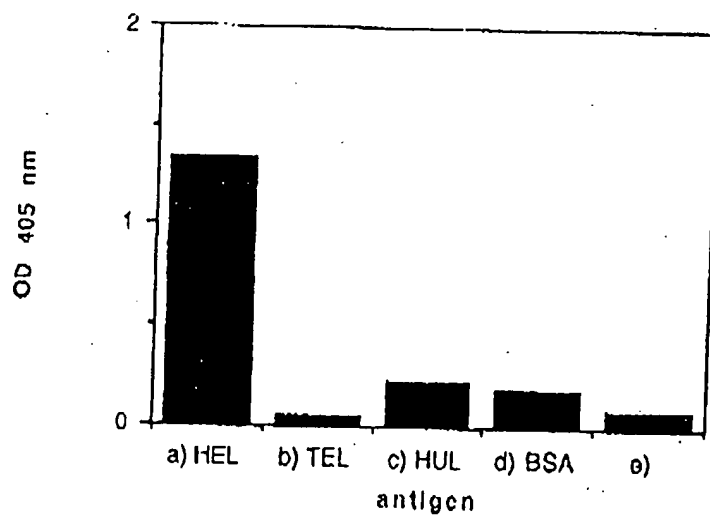
Cleavage site

```
                              Q   V   Q   L   Q         L   E   I   K   R
TAT TCT CAC AGT GCA CAG GTC CAA CTG CAG GAG CTC GAG ATC AAA CGG
            Apa LI                  Pst I        Xho
```

```
GCG GCC GCA GAA ACT GTT GAA AGT etc.
    Not I
```

Restriction enyme sites are shown as well as the amino acids encoded by antibody derived sequences. These are flanked at the 5' end by the gene 3 signal peptide and at the 3' end by 3 alanine residues (encoded by the Not 1 restriction site) and the remainder of the mature gene 3 protein.

Figure 9 Binding of pAb(D1.3) to lysozymes

Figure 10: Sequence of Fab D1.3

```
                              M  K  Y  L  L  P  T  A  A  A  G  L  L  L  P  A  A  Q  P  A
CATGCAAATTCTATTTCAAGGAGACAGTCATAATGAAATACCTATTGCCTACGGCAGCCGCTGGATTGTTATTACCTGCTGCCCAACCAG
        11        21        31        41        51        61        71        81        91


   M  A  Q  V  Q  L  Q  E  S  G  P  G  L  V  A  P  S  Q  S  L  S  I  T  C  T  V  S  G  F  S
CGATGGCCCAGGTGCAGCTGCAGGAGTCAGGACCTGGCCTGGTGGCGCCCTCACAGAGCCTGTCCATCACATGCACCGTCTCAGGGTTCT
       101       111       121       131       141       151       161       171       181


   L  T  G  Y  G  V  N  W  V  R  Q  P  P  G  K  G  L  E  W  L  G  M  I  W  G  D  G  N  T  D
CATTAACCGGCTATGGTGTAAACTGGGTTCGCCAGCCTCCAGGAAAGGGTCTGGAGTGGCTGGGAATGATTTGGGGTGATGGAAACACAG
       191       201       211       221       231       241       251       261       271


   Y  N  S  A  L  K  S  R  L  S  I  S  K  D  N  S  K  S  Q  V  F  L  K  M  N  S  L  H  T  D
ACTATAATTCAGCTCTCAAATCCAGACTGAGCATCAGCAAGGACAACTCCAAGAGCCAAGTTTTCTTAAAAATGAACAGTCTGCACACTG
       281       291       301       311       321       331       341       351       361


   D  T  A  R  Y  Y  C  A  R  E  R  D  Y  R  L  D  Y  W  G  Q  G  T  T  V  T  V  S  S  A  S
ATGACACAGCCAGGTACTACTGTGCCAGAGAGAGAGATTATAGGCTTGACTACTGGGGCCAAGGCACCACGGTCACCGTCTCCTCAGCCT
       371       381       391       401       411       421       431       441       451


   T  K  G  P  S  V  F  P  L  A  P  S  S  K  S  T  S  G  G  T  A  A  L  G  C  L  V  K  D  Y
CCACCAAGGGCCCATCGGTCTTCCCCCTGGCACCCTCCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGCTGCCTGGTCAAGGACT
       461       471       481       491       501       511       521       531       541


   F  P  E  P  V  T  V  S  W  N  S  G  A  L  T  S  G  V  H  T  F  P  A  V  L  Q  S  S  G  L
ACTTCCCCGAACCGGTGACGGTGTCGTGGAACTCAGGCGCCCTGACCAGCGGCGTGCACACCTTCCCGGCTGTCCTACAGTCCTCAGGAC
       551       561       571       581       591       601       611       621       631
```

Fig. 10. cont. (1)

```
      Y  S  L  S  S  V  V  T  V  P  S  S  S  L  G  T  Q  T  Y  I  C  N  V  N  H  N  P  S  N  T
⬤ TCTACTCCCTCAGCAGCGTGGTGACTGTGCCCTCCAGCAGCTTGGGCACCCAGACCTACATCTGCAACGTGAATCACAACCCCAGCAACA
         641       651       661       671      ·681       691       701       711       721
```

```
      K  V  D  K  K  V  E  P  K  S  S  *  *                                              M  K
   CCAAGGTCGACAAGAAAGTTGAGCCCAAATCTTCATAATAACCCGGGAGCTTGCATGCAAATTCTATTTCAAGGAGACAGTCATAATGAA
         731   ·    741       751       761       771       781       791       801       811
```

```
      Y  L  L  P  T  A  A  A  G  L  L  L  P  A  A  Q  P  A  M  A  D  I  E  L  T  Q  S  P  A  S
   ATACCTATTGCCTACGGCAGCCGCTGGATTGTTATTACCTGCTGCCCAACCAGCGATGGCCGACATCGAGCTCACCCAGTCTCCAGCCTC
         821     .831       841       851       861       871       881       891       901
```

```
      L  S  A  S  V  G  E  T  V  T  I  T  C  R  A  S  G  N  I  H  N  Y  L  A  W  Y  Q  Q  K  Q
   CCTTTCTGCGTCTGTGGGAGAAACTGTCACCATCACATGTCGAGCAAGTGGGAATATTCACAATTATTTAGCATGGTATCAGCAGAAACA
         911       921       931       941       951       961       971       981       991
```

```
      G  K  S  P  Q  L  L  V  Y  Y  T  T  T  L  A  D  G  V  P  S  R  F  S  G  S  G  S  G  T  Q
   GGGAAAATCTCCTCAGCTCCTGGTCTATTATACAACAACCTTAGCAGATGGTGTGCCATCAAGGTTCAGTGGCAGTGGATCAGGAACACA
        1001      1011      1021      1031      1041     .1051      1061      1071      1081
```

```
      Y  S  L  K  I  N  S  L  Q  P  E  D  F  G  S  Y  Y  C  Q  H  F  W  S  T  P  R  T  F  G  G
   ATATTCTCTCAAGATCAACAGCCTGCAGCCTGAAGATTTTGGGAGTTATTACTGTCAACATTTTTGGAGTACTCCTCGGACGTTCGGTGG
        1091      1101    · 1111      1121      1131      1141      1151      1161   ·  1171
```

```
      G  T  K  L  E  I  K  R  T  V  A  A  P  S  V  F  I  F  P  P  S  D  E  Q  L  K  S  G  T  A
   AGGCACCAAGCTCGAGATCAAACGGACTGTGGCTGCACCATCTGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAACTGC
        1181    . 1191      1201      1211      1221      1231      1241      1251      1261
```

Fig. 10. cont. (2).

```
   S   V   V   C   L   L   N   N   F   Y   P   R   E   A   K   V   Q   W   K   V   D   N   A   L   Q   S   G   N   S   Q
CTCTGTTGTGTGCCTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACAGTGGAAGGTGGATAACGCCCTCCAATCGGGTAACTCCCA
     1271       1281       1291       1301       1311       1321       1331       1341       1351
```

```
   E   S   V   T   E   Q   D   S   K   D   S   T   Y   S   L   S   S   T   L   T   L   S   K   A   D   Y   E   K   H   K
GGAGAGTGTCACAGAGCAGGACAGCAAGGACAGCACCTACAGCCTCAGCAGCACCCTGACGCTGAGCAAAGCAGACTACGAGAAACACAA
     1361       1371       1381       1391       1401       1411       1421       1431       1441
```

```
   V   Y   A   C   E   V   T   H   Q   G   L   S   S   P   V   T   K   S   F   N   R   G   E   S   *   *
AGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGTTCGCCCGTCACAAAGAGCTTCAACCGCGGAGAGTCATAGTAAGGATCCAGCTC
     1451       1461       1471       1481       1491       1501       1511       1521       1531
```

GAATTC

FabD1.3 in
pUC19

Fig. 10 cont. (3).

Fig. 11 Comparison of lysozyme-binding by phage-Fab and phage -ScFv

O.D. 405nm

Vector
Fd ScFv (OX)
Fd VHCH1 (D1.3)
Fd Fab (D1.3)
Fd ScFv (D1.3)

Fig. 12

Figure 13 Sequence of pNQ11ScFv

```
 Q   V   Q   L   Q   E   S   G   G   G   L   V   Q   P   G   G
CAG GTG CAG CTG CAG GAG TCA GGA GGA GGC TTG GTA CAG CCT GGG GGT
            PstI

 S   L   R   L   S   C   A   T   S   G   F   T   F   S   N   Y
TCT CTG AGA CTC TCC TGT GCA ACT TCT GGG TTC ACC TTC AGT AAT TAC

 Y   M   G   W   V   R   Q   P   P   G   K   A   L   E   W   L
TAC ATG GGC TGG GTC CGC CAG CCT CCA GGA AAG GCA CTT GAG TGG TTG

 G   S   V   R   N   K   V   N   G   Y   T   T   E   Y   S   A
GGT TCT GTT AGA AAC AAA GTT AAT GGT TAC ACA ACA GAG TAC AGT GCA

 S   V   K   G   R   F   T   I   S   R   D   N   F   Q   S   I
TCT GTG AAG GGG CGG TTC ACC ATC TCC AGA GAT AAT TTC CAA AGC ATC

 L   Y   L   Q   I   N   T   L   R   T   E   D   S   A   T   Y
CTC TAT CTT CAA ATA AAC ACC CTG AGA ACT GAG GAC AGT GCC ACT TAT

 Y   C   A   R   G   Y   D   Y   G   A   W   F   A   Y   W   G
TAC TGT GCA AGA GGC TAT GAT TAC GGG GCC TGG TTT GCT TAC TGG GGC

 Q   G   T   L   V   T   v   s   s   g   g   g   g   s   g   g   g   g   s
CAA GGG ACC CTG GTC ACC gtc tcc tca ggtggaggcggttcaggcggaggtggctct
            BstEII

 g   g   g   g   s   d   i   E   L   T   Q   T   P   L   S   L   P   V
ggcggtggcggatcggac atc GAG CTC ACC CAA ACT CCA CTC TCC CTG CCT GTC
                       SacI

 S   L   G   D   Q   A   S   I   S   C   R   S   S   Q   S   I
AGT CTT GGA GAT CAA GCC TCC ATC TCT TGC AGA TCT AGT CAG AGC ATT

 V   H   S   N   G   N   T   Y   L   E   W   Y   L   Q   K   P
GTA CAT AGT AAT GGA AAC ACC TAT TTA GAA TGG TAC CTG CAG AAA CCA
                                                    PstI

 G   Q   S   P   K   L   L   I   Y   K   V   S   N   R   F   S
GGC CAG TCT CCA AAG CTC CTG ATC TAC AAA GTT TCC AAC CGA TTT TCT

 G   V   P   D   R   F   S   G   S   G   S   G   T   D   F   T
GGG GTC CCA GAC AGG TTC AGT GGC AGT GGA TCG GGG ACA GAT TTC ACA

 L   K   I   S   R   V   E   A   E   D   L   G   V   Y   Y   C
CTC AAG ATC AGC AGA GTG GAG GCT GAG GAT CTG GGA GTT TAT TAC TGC

 F   Q   G   S   H   V   P   Y   T   F   G   G   G   T   K   L
TTT CAA GGT TCA CAT GTT CCG TAC ACG TTC GGA GGG GGG ACC AAG CTC

 E   I   K   R
GAG ATC AAA CGG
XhoI
```

Restriction sites refered to in the text are shown underlined. The sequence contributed by the linker is shown in lower case.
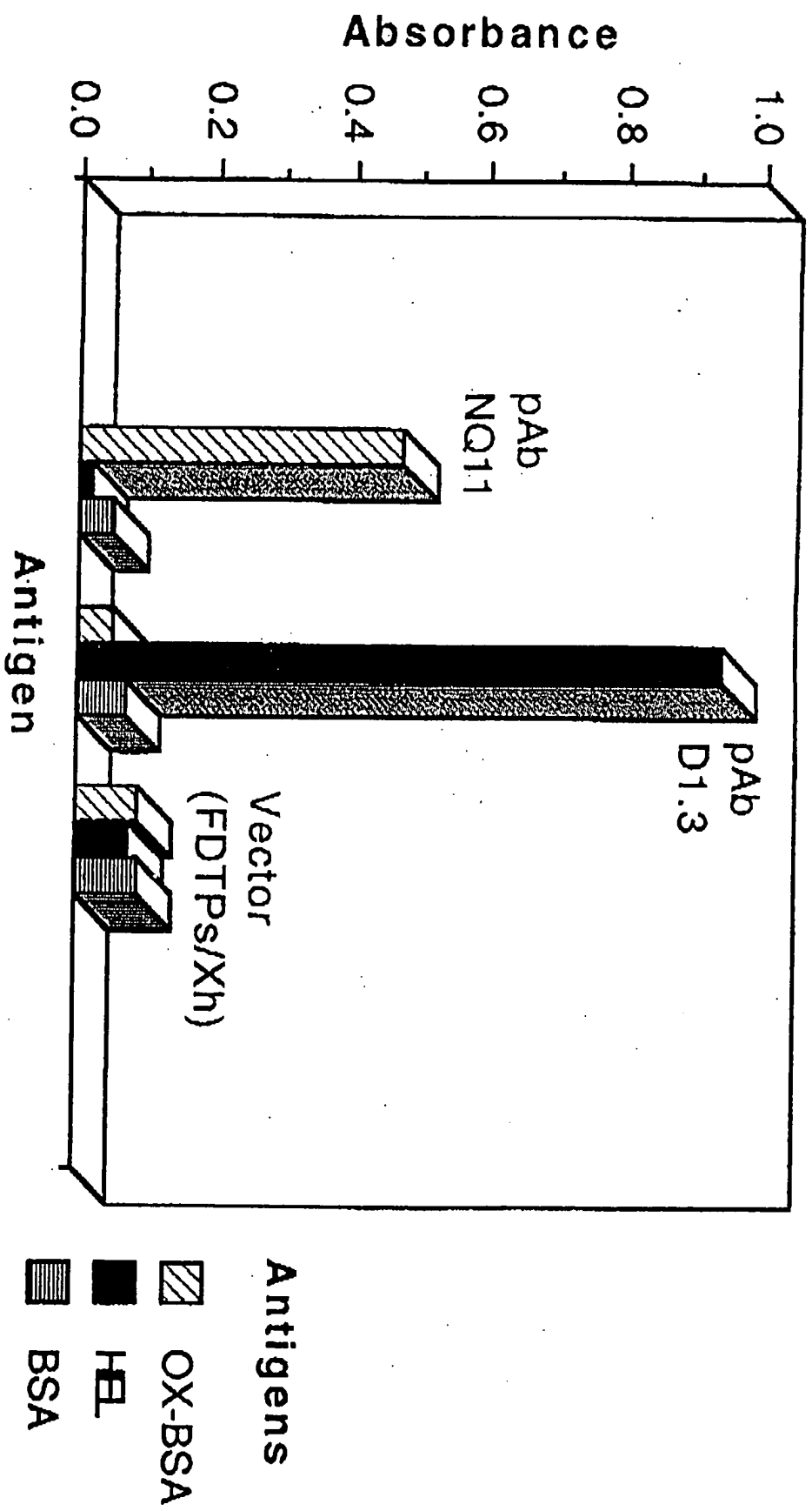
Figure14.Binding of pAbs to specific antigens

Fig. 15. Sequence surrounding phoA insertion in fd-phoA1

SEQUENCE AT 5' END OF phoA INSERTION IN fd-phoA1

```
                                                  R   T   P   E   M   P   V   L
Signal peptide
cleavage site
       ▼
TCT CAC AGT GCA CAA ACT GTT GAA CGG ACA CCA GAA ATG CCT GTT CTG
         ApaLl
```

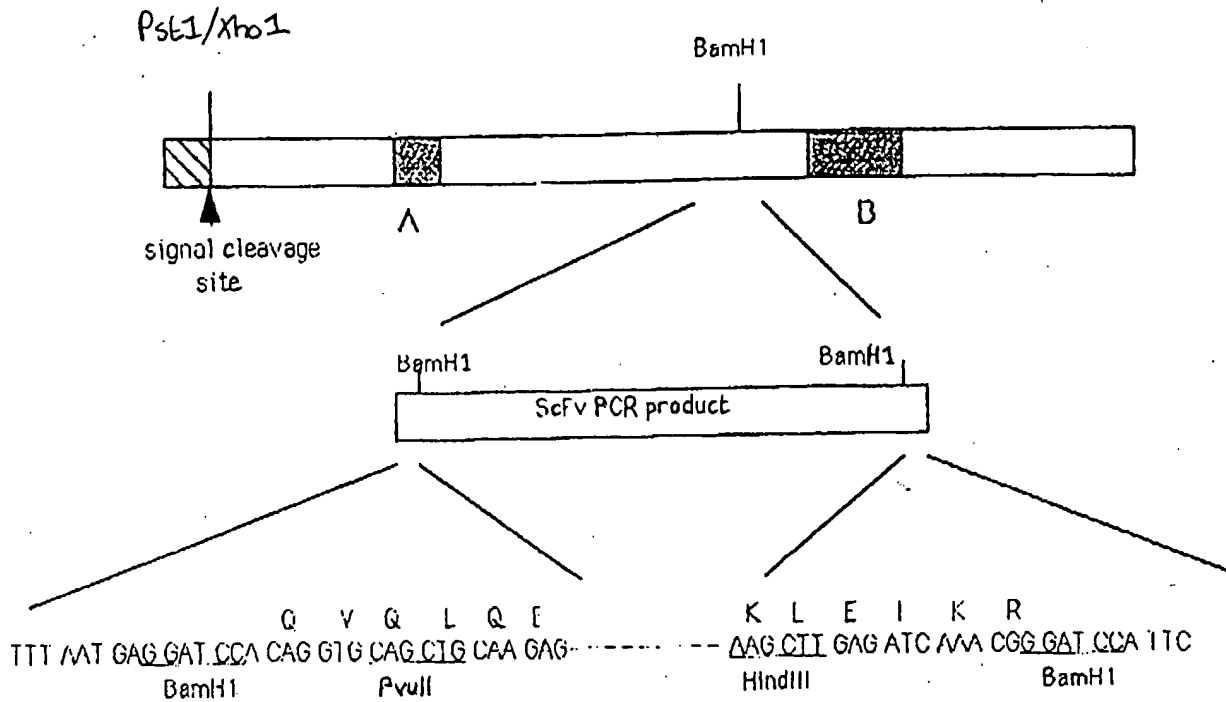## SEQUENCE AT 3' END OF phoA INSERTION IN fd-phoA1

```
 K   A   A   L   G   L   K
AAA GCC GCT CTG GGG CTG AAA GCG GCC GCA GAA ACT GTT GAA AGT etc.
                              NotI
```

The restriction sites used for cloning are shown as well as the as the amino acids encoded by phoA around the insertion site. In this example, the first five amino acids of the mature fusion will actually come from gene 3.

Figure 16. Structure of gene 3

1)

Pst1/Xho1

BamH1

signal cleavage site

A

B

BamH1   BamH1

ScFv PCR product

    Q   V Q   L   Q E   K   L   E   I   K   R

TTT AAT GAG GAT CCA CAG GTG CAG CTG CAA GAG------ -- AAG CTT GAG ATC AAA CGG GAT CCA TTC

BamH1   Pvull   HindIII   BamH1

2)   **A**   (1834) 5' GAG GGT GCT GGC TCT

                  "   "   "C "   "

                  "   "   "C "   "

                  "   "   "C " ACT 3'(1839)

    B   (2284) 5' -   GGC GGC GGC TCT

                  -- GGT CGT GGT "

                    " GGC GGC "

              GAG "   " GGC "

                "   "   " GGT "

                "   "   " GGC "

                "   "   " GGT "

                "   "   " GGC " 3'(2379)

Reverse complement of mutagenic oligo G3Bamlink

                              T

5'   CAG GGT GGC GGA TCC

                   T

GAG GGT GGC GG 3'

Numbering is according to Beck et al (1978, supra).

Fig. 17

VH1BACK→   VK2BACK→

←VHIFOR-2   ←MJK1(2,4,5)FNOX

↓ PRIMARY PCR FROM cDNA

Heavy   Kappa

↓ MIX PRODUCTS WITH LINKER DNA
(MADE BY PCR)

←MJK1(2,4,5)FNOX

Primary PCR
products

linker

VH1BACK→

↓ SECOND ASSEMBLY PCR
USING 'OUTER' PRIMERS

←JK1(2,4,5)NOT10

Assembled combinatorial
product

HKAPA10 →

↓ PULL-THROUGH (tagged primers)

ApaLI   NotI

↓ DIGEST AND CLONE

Fig. 18

Figure 19

Figure 20 Displacement by unlabelled PDGF

Figure 21 PGDF displacement vector binding deducted

Figure 22: Elisa of lysozyme binding by pCAT-3 ScFv D1.3 phagemid in comparison with pCAT-3 vector (both rescued by M13KO7) and fdCAT2 ScFvD1.3 as described in example 17. ELISA was performed as described in example 6 with modifications detailed in example 17.

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